Investigations on the Anti-diabetic Activities of Traditional Chinese Medicine Formulae Originally Used against Diabetic Foot Ulcer

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A Thesis Submitted in Partial Fulfilment
of the Requirements for the Degree of

Master of Philosophy
in
Biochemistry

July, 2004


香港中文大學
圖書館系統
12 APR 2005
THE CHINESE UNIVERSITY OF HONG KONG

Abstract

Diabetes mellitus is a metabolic disorder marked by chronic hyperglycaemia. Around 90% of the cases are diagnosed as type 2 diabetes. Insulin resistance and impaired insulin secretion are the two main features of the disorder. Complications of diabetes impose major public health burdens worldwide, for example, about 15% of the patients develop foot ulcer. In a clinical study, two traditional Chinese medicine formulae, 托毒生肌顆粒劑 (formula 1) and 耆味地黃顆粒劑 (formula 2), have saved over 80% of the diabetic foot ulcer patients from amputation. We would like to explode the possibilities that the formulae and their individual components may possess anti-diabetic activities since glycaemic control of the foot ulcer patients is one of the effective ways to treat the ulcer. The studies in this project test the hypothesis that formula 1 and its six component herbs have anti-diabetic activities. The six component herbs of formula 1 were firstly authenticated by their morphological characteristics, and further supported by thin layer chromatographic profiles. The herbal extracts were then screened for their effects on: (1) Glucose uptake in 3T3-L1 adipocytes and Hs68 skin fibroblasts; (2) Gluconeogenesis in H4IIE hepatoma cells; (3) Glucose absorption in brush border membrane vesicles. The screening results indicated that formula 1 and its component herb, Rhizoma Smilacis Chinensis (菝葜), are highly potent on regulating cellular glucose homeostasis. These two herbal extracts were further tested for their *in vivo* anti-diabetic activities using a streptozotocin(STZ)-induced type 2 diabetic rat model. The results showed that formula 1 and Rhizoma Smilacis Chinensis did not significantly improve the oral glucose tolerance and basal glycaemia in the diabetic rats. The effects of formula 1 and formula 2 on glucose homeostasis were also studied in

diabetic patients. Preliminary results showed that the formulae did not significantly improve fasting plasma glucose level or erythrocyte glucose transport. In conclusion, a systematic approach has been developed and used to investigate the anti-diabetic activities of traditional Chinese medicine. The results indicate that formula 1 and its component herbs modulate glucose homeostasis at the tissue levels without improving systemic glycaemic control. From our results, we can tell that it is safe for the patients to receive both formulae and medication treatment without worrying about the side effect of hypoglycaemia.

Abstract in Chinese 摘要

治療糖尿病足潰瘍中藥方劑的抗糖尿病作用研究

糖尿病是一種以高血糖爲特徵的代謝疾病, 大概百分之九十的糖尿病病症被証實爲二型糖尿病。胰島素抵抗及胰臟減少胰島素分泌爲二型糖尿病的兩大特徵。 糖尿病的併發症爲公眾健康帶來沈重負擔, 例如, 百分之十五的糖尿病病人會併發糖尿病足潰瘍。在一項初部臨床試驗中, 兩條傳統中藥方劑, 托毒生肌顆粒劑 (F1) 及耆味地黃顆粒劑 (F2), 免除了超過百分之八十的糖尿病足潰瘍病人進行截肢手術的必要, 所以我們希望可以研究這些方劑有否進一步的抗糖尿病作用。本研究假設 F1 及其六種單味擁有抗糖尿病的作用。在研究它們的抗糖尿病活性之先, 我們以形態學分析及薄層層析的分法去鑑定 F1 的六種單味。然後, 我們以數個和調節體內血糖平衡機制相關的細胞及試管實驗模型去測試 F1 及其六種單味的水提取物的抗糖尿作用及其機理: (一) 3T3-L1 脂肪細胞及 Hs68 纖維細胞的葡萄糖攝入; (二) H4IIE 肝癌細胞的糖異生作用; (三) 小腸刷狀緣囊泡的葡萄糖吸收。研究發現 F1 及其單味, 菝葜, 在以上各種測試中的效果突出。我們對這兩種水提取物進行動物測試以研究它們在體內的抗糖尿病作用, 爲此我們採用了對初生大白鼠注射鏈脲佐菌素所引致的二型糖尿病大白鼠實驗模型。 結果發現這兩種中藥都對基礎血糖及口服葡萄糖耐量的改善沒有顯著效果。我們亦研究了 F1 及 F2 對糖尿病病人的體內血糖平衡的影響, 初部研究結果發現這兩條中藥方劑並沒有顯著地改善病人的空腹血糖或紅細胞葡萄糖攝取之活性。總括而言, 本研究採用了系統化的方法來研究中藥的抗糖尿病活性, 利用這研究平台我們發現, F1 及其單味可以調節身體組織的葡萄糖平衡, 但並無

降血糖的作用。但我們可以說那些現正服食這方劑的病人不會受低血糖的副作用影響。

Acknowledgements

This work would not have been possible without the support and encouragement of my supervisors, Prof. K. P. Fung, Prof. Y.Y. Ho and Prof. Clara B.S. Lau. I am grateful for their mentoring and guidance throughout the project and thesis preparation. I would also like to thank Prof. P.C. Leung for his supports and advices.

I would like to make special thanks to our research team members, Ms. K.M. Lau, Ms. Y.W. Chan, Mr. T.W. Lau, Mr. C.H. Lau and Ms. F.C. Lam for their assistance in the animal studies. I am grateful to Ms. Y.S. Fong, Ms. W.M. Lo and L.Y. Wong for their help on handling of the blood samples in the clinical studies. I am also thankful to all staff of the Institute of Chinese Medicine, CUHK, especially Ms. K.L. Choi and Ms. S.W. Cheng for their assistance in the TLC and HPLC studies. I would also like to thank Ms. Stacey Ng for her help on thesis writing.

I would like to thank Dr. H. Cao of the National Engineering Research Center for Modernization of TCM (Zhuhai, China) for morphological authentication of herbs; Dr. K.C. Choi at the School of Public Health, CUHK, for the statistical analysis; Dr. Anthony E. James and his colleagues in the Laboratory Animal Service Centre, CUHK, for providing support towards animal studies; and all other people who have taken part in this project. Finally, I cannot end without thanking my family and friends, who gave me constant encouragement throughout my study.

The work described in this thesis was substantially supported by a grant from the University Grants Committee of the Hong Kong Special Administrative Region, China under the Area of Excellence project 'Chinese Medicine Research and Further Development' (Project No. AoE/B-10/01) coordinated by the Institute of Chinese Medicine of The Chinese University of Hong Kong.

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Abbreviations

2-DG	2-Deoxy-D-glucose
3-OMG	3-O-Methyl-D-gluco-pyranose
ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
AUC	Area under the curve
BBMV	Brush border membrane vesicles
BCA	Bicinchoninic acid
BMI	Body mass index
BSA	Bovine serum albumin
DEX	Dexamethasone
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELSD	Evaporative light scattering detector
FBS	Fetal bovine serum
FFA	Free fatty acids
Formula 1/F1	托毒生肌顆粒劑
Formula 2/F2	耆味地黃顆粒劑
G6Pase	Glucose-6-phosphatase
GDP	Guanosine 5'-diphosphate
GFP	Green fluorescence protein
Glut1	Glucose transporter 1
Glut2	Glucose transporter 2
Glut3	Glucose transporter 3
Glut4	Glucose transporter 4
Glut5	Glucose transporter 5
HbA1c	Haemoglobin A1c

H/C	Hot/cold mixture
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HPLC	High performance liquid chromatography
i.p.	Intraperitoneal
i.v.	Intravenous
IBMX	3-Isobutyl-1-methylxanthine
IDDM	Insulin-dependent diabetes mellitus
LDL	Low-density lipoprotein
MODY	Maturity-onset diabetes of the young
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NIDDM	Non-insulin-dependent diabetes mellitus
PBS	Phosphate-buffered saline
pCPT-cAMP	8-(4-chlorophenylthio)-cAMP
PEPCK	Phosphoenolpyruvate carboxykinase
PS	Penicillin-Streptomycin
R_f	Retardation factor
SEM	Standard error of mean
Sglt1	Na^+-dependent glucose transporter 1
STZ	Streptozotocin
TCM	Traditional Chinese medicine
TLC	Thin layer chromatography
TNF-α	Tumor Necrosis Factor alpha
Tris	Tris(hydroxymethyl)aminomethan
UV	Ultraviolet light
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight
WHO	World Health Organization

Chapter 1: Introduction

1.1. Definition of diabetes mellitus

Diabetes is a major health burden in the world. According to the World Health Organization (WHO), it is estimated that there were about 171 million diabetes cases worldwide in 2000 and the number of cases is estimated to increase to 366 million by year 2030 (Wild *et al.*, 2004). In Hong Kong, diabetes mellitus is also a prevalent disorder. About 10% of the population is suffering from diabetes (Diabetes Division, Hong Kong Society for Endocrinology, Metabolism, and Reproduction, 2000), and the number is still increasing.

Diabetes mellitus is a metabolic disorder, which is characterized by the presence of chronic hyperglycaemia, and it is caused by insufficient insulin production from the pancreatic ß-cells and/or ineffectiveness of insulin action of the target tissues (World Health Organization, 1999).

The diagnosis criteria of diabetes were revised by American Diabetes Association in 2002 (American Diabetes Association., 2004), which are shown in Table 1.1. The diagnosis is based on the measure of hyperglycaemia. Three criteria are considered, including casual plasma glucose level, fasting plasma glucose and 2-hour post glucose load plasma glucose during oral glucose tolerance test. The new diagnostic criteria state that any individual who fits the first criterion with diabetic symptoms or the second and third criteria being asymptotic is considered as a diabetic patient. Symptoms of diabetes include polyuria (excessive passage of urine), polydipsia (excessive thirst), weight loss, sometimes with polyphagia (excessive desire to eat), and ketoacidosis (acidosis with an accumulation of ketone bodies).

Despite the use of a single factor of diagnosing diabetes, which is the measurement of hyperglycaemia, the aetiology and clinical presentations of diagnosed diabetic patients may be different. These differences among patients gave rise to the classification of diabetes, which is described in section 1.2.

	Casual plasma glucose	Fasting plasma glucose	Oral glucose tolerance test (2hr post glucose load)
Normal	---	≤100mg/dl (≤5.5mM)	<140 mg/dl (<7.7mM)
Impaired glucose tolerance	---	100-125 mg/dl (5.6 – 6.9mM)	140-199 mg/dl (7.7 – 11.1mM)
Diabetes	≥200 mg/dl (≥11.1mM)	≥126 mg/dl (≥7.0mM)	≥200 mg/dl (≥11.1mM)

Table 1.1. Diagnostic values of diabetes mellitus and other categories of hyperglycaemia, according to criteria published by American Diabetes Association. Casual plasma glucose is defined as the plasma glucose level at any time of day. Fasting is defined as no caloric intake for at least 8 hours. Oral glucose tolerance test should be performed by using a glucose load containing the equivalent of 75g glucose dissolved in water (American Diabetes Association, 2004).

1.2. Classification of diabetes mellitus

Diabetes mellitus was previously classified into insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM). However, this classification did not consider the specific cause or aetiology of the disorder. In 1999, the classification of diabetes was revised by WHO and the new classification reflects the stages of hyperglycaemia, as well as the aetiology. Diabetes mellitus is now classified into mainly two types: type 1 and type 2 diabetes, which are different in aetiology, pathophysiology and clinical manifestation. Apart from the usual type 1 and type 2 diabetes, there are also some less frequently occurring types of diabetes, including gestational diabetes, maturity-onset diabetes of the young (MODY) and diabetes secondary to other diseases (World Health Organization, 1999).

1.2.1. Type 1 diabetes

Type 1 diabetes, formerly known as IDDM or juvenile-onset diabetes, is caused by the pancreatic β-cells destruction and the failure of the pancreas to produce insulin. Patients with type 1 diabetes are prone to ketoacidosis and rely on insulin injection for survival. This form of diabetes develops most frequently in children and adolescents. Type 1 diabetes is regarded as an autoimmune disease that causes the destruction of ß-cells of the Islet of Langerhans of pancreas (Mathis *et al.*, 2001) by activated lymphocytic T cells (Conget, 2002), resulting in insufficient insulin secretion and hyperglycaemia. Type 1 diabetes is characterized by the presence of autoantibodies to insulin, islet cells or glutamic acid decarboxylase (Winter *et al.*, 2002). It is found that genetic factors may contribute to the susceptibility of individuals to the disorder, for example, the presence of certain halotypes in HLA genes on chromosome 6 (Conget, 2002). Epidemiological studies

also suggest that the disorder is related to environmental factors, which are still poorly defined (Zimmet *et al.*, 2001). However, few cases of type 1 diabetes, which are prone to ketoacidosis and require insulin for survival, do not exhibit autoimmune destruction of the pancreas. These patients are called "Type 1 idiopathic diabetes". These cases have no known aetiologies and they are strongly inherited, but with no evidence of autoimmune destruction (Tiberti *et al.*, 2000).

1.2.2. Type 2 diabetes

Type 2 diabetes, formerly known as NIDDM or maturity-onset diabetes, results from the body's inability to respond properly to the action of insulin produced by the pancreas and the inability of the β-cells to secrete enough insulin to overcome the inadequate response. It occurs most frequently in adults and it is the most prevalent form of diabetes, which accounts for around 90% of all diabetes cases worldwide (Williamson *et al.*, 2004). This type of diabetes is closely associated with affluent lifestyle and overweight. Patients with type 2 diabetes are characterized by the presence of insulin resistance and impaired insulin secretion. Insulin resistance is the reduced sensitivity of the insulin target tissues to the normal circulating level of insulin. It results in the decreased glucose disposal in muscle and adipose tissue and the lack of suppression on hepatic glucose output upon insulin action (Goldstein, 2003), which in turn create a tendency to increase the blood glucose level. To maintain normoglycaemia, an increased amount of insulin is released by the pancreatic β-cells, resulting in hyperinsulinaemia. The co-occurrence of insulin resistance and hyperinsulinaemia characterizes the impaired glucose tolerance state, which precedes type 2 diabetes (Pirola *et al.*, 2003). However, sustained hyperinsulinaemia would cause impairment in insulin secretion and action

(Ostenson, 2001), and result in pancreatic ß-cell defects and impaired insulin secretion. The decrease in insulin secretion under the state of insulin resistance means that the plasma insulin level can no longer sustain normal glucose level and it results in hyperglycaemia and hence, type 2 diabetes (Figure 1.1).

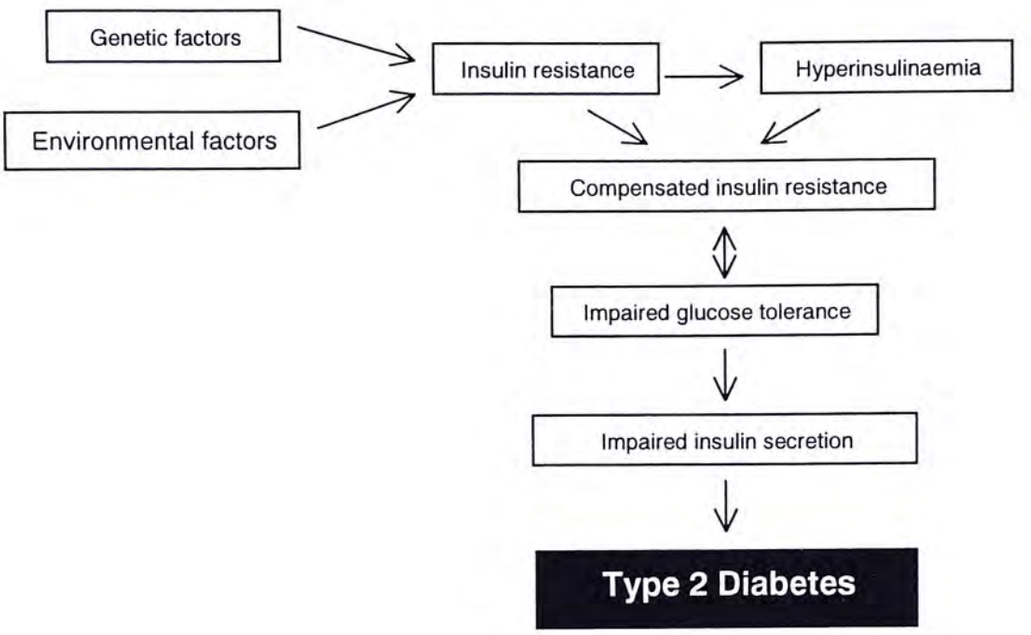


Figure 1.1. The natural history of type 2 diabetes mellitus. Insulin resistance is predisposed by genetic and environmental factors. To maintain normoglycaemia in insulin resistant state, hyperinsulinaemia is needed. Sustained hyperinsulinaemia results in insulin secretion and action impairment and hence, pancreatic ß-cells defects and impaired insulin secretion. Impaired insulin secretion, together with insulin resistance, cause hyperglycaemia and therefore, type 2 diabetes (Ostenson, 2001).

Type 2 diabetes is closely related to both genetic and environmental factors (Figure 1.1). Although diabetes caused by mutation of a single gene is identified, such as MODY, type 2 diabetes seems to be a polygenic disorder, rather than monogenic (Froguel and Velho, 2001). However, the genes involved in the pathogenesis of this disorder remain unidentified. Epidemiological studies found that the prevalence of type 2 diabetes is very high in certain ethnic groups, such as the American Pima Indians, Australian Aborigines and Pacific Islanders (Zimmet *et al.*, 2001). Studies among identical twins with type 2 diabetes show that the concordance rate can be as high as 90% (Lo *et al.*, 1991; Medici *et al.*, 1999). These evidences suggest that diabetic phenotype is affected by genetic factors of the individuals. It is also generally known that type 2 diabetes segregates in families in an inherited condition. An offspring with one of the parents having type 2 diabetes has 3.5-fold greater risk to get the disorder when compared with another offspring without parental diabetes, while those with both parents having this disorder has a 6-fold higher risk (Meigs *et al.*, 2000).

Most cases of type 2 diabetes are strongly associated with sedentary lifestyle and obesity (Zimmet, 1999). Over 90% of the newly diagnosed patients are obese. Body mass index (BMI) is a strong predictor for type 2 diabetes. Research showed that the relative risk of developing diabetes increased linearly with BMI. A BMI of 23-25 can increase the risk of diabetes by 3-fold compared with a BMI of less than 21 (Carey *et al.*, 1997). Individuals with BMI higher than 35 have a 30–40-fold increased risk of diabetes (Adler, 2002). Obesity is a major factor of insulin resistance (Kahn and Flier, 2000). Insulin resistance occurs early in the development of obesity, long before any occurrence of diabetes. It is found

that insulin secretion is elevated in the course of an oral glucose tolerance test in obese subjects and the rise of insulin represents a compensatory mechanism for maintaining normoglycaemia. Insulin resistance is demonstrated by the reduction of non-oxidative glucose disposal (Felber and Golay, 2002). Elevated plasma free fatty acids (FFA) level is also a major factor contributing insulin resistance in obesity. Plasma FFA induces insulin resistance through inhibition of cellular glucose disposal (Dresner *et al.*, 1999).

With the change of the lifestyle in most developed and developing countries, over-nutrition and sedentary lifestyle become more common. More type 2 diabetes cases are expected and it will continue to impose an increasing burden on the healthcare system in the future.

1.2.3. Other forms of diabetes

Apart from type 1 and type 2 diabetes, there are several less common forms of diabetes. Table 1.2 summarizes these forms of diabetes. The causes of these forms of diabetes are in a large variation, from genetic defects to induction by drugs or infections. However, these cases constitute only a small proportion of the whole diabetes population (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003).

Aetiology Types	Examples
Genetic defects of β-cell function	Mitochondrial DNA mutation
	Chromosome 20, HNF-4α (MODY1)
	Chromosome 7, glucokinase (MODY2)
Genetic defects in insulin action	Type A insulin resistance
	Leprechaunism
Diseases of the exocrine pancreas	Pancreatitis
	Cystic fibrosis
	Trauma/pancreatectomy
Endocrinopathies	Acromegaly
	Cushing's syndrome
	Glucagonoma
Drug- or chemical-induced	Vacor
	Thyroid hormone
Infections	Congenital rubella
Uncommon forms of immune-mediated diabetes	Anti-insulin receptor antibodies
Other genetic syndromes sometimes associated with diabetes	Down's syndrome
	Klinefelter's syndrome
	Turner's syndrome
Gestational diabetes mellitus	

Table 1.2. The summary of other forms of diabetes mellitus. (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003).

1.3. Complications of diabetes mellitus

Cardiovascular complications, retinopathy, nephropathy, neuropathy and foot ulcer are the most common complications of diabetes.

Cardiovascular complications, such as coronary heart disease and strokes, are the most common cause of morbidity and mortality in diabetes. Hyperglycaemia promotes the formation of advanced glycation products, which cross-link with collagen, causing arterial stiffness. Dyslipidaemia in diabetes causes increased levels of low-density lipoprotein (LDL) cholesterol and promotes atherogenesis (Bate and Jerums, 2003). Hypertension is more common as is a hypercoaguable state.

Retinopathy causes blindness in diabetic patients by causing hemorrhages and angiogenesis in the retina, resulting in damaged vision. Diabetes also accelerates cataract formation. Studies have suggested that eventually up to 75% of diabetic patients will develop retinopathy of some forms (Ewing *et al.*, 1998).

Nephropathy is a syndrome of albuminuria, declining glomerular filtration rate and arterial hypertension that affects 20–40% of the diabetic patients (Ruggenenti and Remuzzi, 2000).

Neuropathy is a progressive deterioration of neuronal functions, resulting in peripheral and autonomic nerve dysfunction, causing painful syndrome, numbness, diarrhea, postural hypotension and erectile dysfunction (Watkins *et al.*, 2003). More than half of diabetic patients develop neuropathy (Feldman, 2003) and it is clear that impaired blood flow and endoneurial hypoxia are the major causes (Cameron *et al.*, 2001).

Foot ulcer and amputation are a major cause of morbidity, disability, as well as emotional and physical costs for diabetic patients (Frykberg *et al.*, 1998). About 15% of the patients will develop a foot ulcer at some point in their lives (Edmonds and Foster,

2004). Foot ulcer is associated with neuropathy and insufficient arterial supply. Infection also aggravates the ulcer condition (Calhoun *et al.*, 2002). Current therapy of this complication is mainly by antibiotics and intensive wound care (Mason *et al.*, 1999).

1.4. Current treatment of diabetes mellitus

1.4.1. Type 1 diabetes

Insulin injection is the major form of treatment of type 1 diabetes (Atkinson and Eisenbarth, 2001). Most individuals in developed countries are now treated with recombinant human insulin. Insulin analogues are also available for the treatment (Garg *et al.*, 1999). Regular insulin needs to be given before meals (i.e., 30 minutes to 1 hour before) (Atkinson and Eisenbarth, 2001).

Insulin therapy has improved the quality of life and extended the life expectancy of patients with type 1 diabetes (Rayat *et al.*, 1999). Hypoglycaemia is the most common adverse effect of the insulin injection (Bolli, 1999). The major risk factor for severe hypoglycaemia is hypoglycaemia unawareness, which occurs particularly in patients with type 1 diabetes of long duration and in those with a history of frequent episodes of hypoglycaemia (Chiarelli *et al.*, 1999).

Islet transplantation is another treatment strategy for type 1 diabetes. Islet transplantation aims to overcome the lifelong need for daily insulin injections and frequent blood glucose testing, to promote maintenance of near normal blood glucose levels, and to avoid or even reverse the long-term diabetes-associated complications (Hirshberg *et al.*, 2003). However, the success rate of islet

transplantation is low. Failures are mostly due to chronic rejections and recurrence of autoimmunity. Also, the islet may be damaged by chronic immunosuppressive therapy (Lohmann *et al.*, 2002).

1.4.2. Type 2 diabetes

1.4.2.1. Diet and exercise

Obesity is a strong predictor for type 2 diabetes. Even a modest degree of overweight can increase the risk of getting diabetes. Therefore, it is not surprising that dietary intervention, weight loss and exercises can effectively ameliorate the diabetic condition. Studies show that moderate weight loss and increased physical activity can increase insulin sensitivity (Kelley *et al.*, 2004; Jequier, 1984). From the Finnish study, it showed that the intensive lifestyle intervention in diet and exercise behaviour reduced diabetes risk and improved glycemia and lipemia (Lindstrom *et al.*, 2003). The Diabetes Prevention Program, which is a large, randomized clinical trial involving adults in the United States who were at high risk for the development of type 2 diabetes, also demonstrated that lifestyle intervention reduced the incidence of diabetes by 58% and metformin by 31% as compared with placebo, which showed that the lifestyle intervention was significantly more effective than metformin (Knowler *et al.*, 2002).

1.4.2.2. Medication

Generally, the above weight loss measures are insufficient or fail to control the diabetic condition. Therefore, medication is necessary for glycaemic control in type 2 diabetic patients. Medication for diabetes treatment not only aims at the pancreas but also the other tissues concerning glucose homeostasis, including

intestine, liver and peripheral tissues. Figure 1.2 illustrates the four target tissues and their respective mechanisms on diabetes treatment (Heine, 1999). The target mechanisms for a better glycaemic control in type 2 diabetes include:

(1) Stimulation of insulin secretion from the pancreas;

(2) Improvement in glucose transport into the peripheral tissues to improve insulin sensitivity of these tissues;

(3) Reduction of the hepatic glucose production;

(4) Inhibition of intestinal glucose absorption.

By the medication, insulin resistance can be ameliorated by the improvement in peripheral glucose transport and the reduction of the hepatic glucose production. Stimulation of insulin secretion from the pancreas can improve both insulin resistance and impaired insulin secretion while inhibition of intestinal glucose absorption directly prevents the postprandial upsurge of blood glucose level. In the following sections, medications concerning these mechanisms are described in details.

Apart from the above four mechanisms, there are some minor target mechanisms for diabetes treatment. For example, some drugs can increase the sense of satiety to the patients to control their appetite.

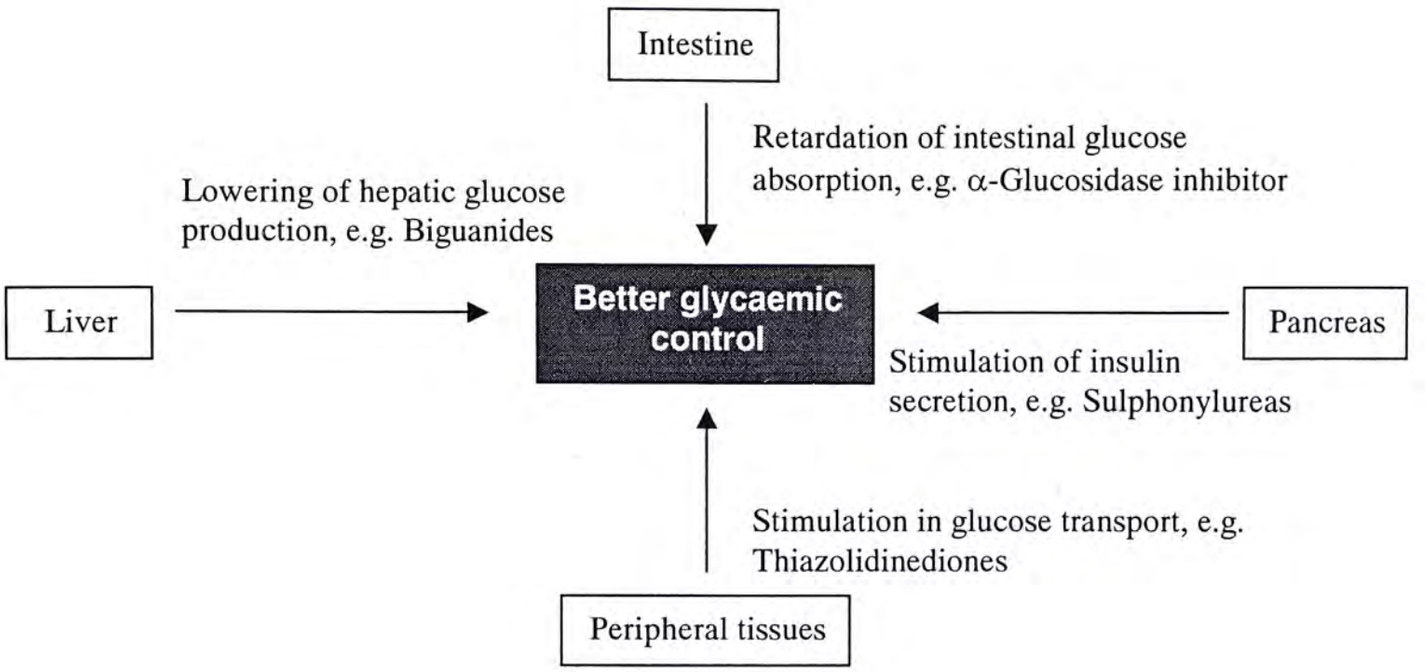


Figure 1.2. The four major targets for type 2 diabetes treatment. The target mechanisms for glycaemic control in type 2 diabetes include stimulation of insulin secretion from the pancreas, improvement in glucose transport into the peripheral tissues, inhibition of the hepatic glucose production and inhibition of intestinal glucose absorption (Heine, 1999).

i. Sulphonylureas

Sulphonylureas can stimulate pancreatic insulin secretion via specific sulphonylurea receptors on the pancreatic ß-cells. Such binding results in depolarization of the plasma membrane and stimulates the release of insulin by moving the insulin-containing granules to the membrane (Chehade and Mooradian, 2000).

One of the most frequent side effects of sulphonylureas treatment is long-term hypoglycaemia, which may cause neuronal defect or death. Another possible side effect is the body weight gain.

ii. Biguanides

The use of biguanides in diabetes treatment can be traced back to the use of *Galega officinalis* (goat's-rue or French lilac) as a treatment for diabetes in medieval Europe. Guanidine, which is the active compound of the plant, was used as the precursor to produce biguanides (Bailey and Day, 1989). The most commonly used biguanides in diabetic treatment in recent years is metformin (Figure 1.3).

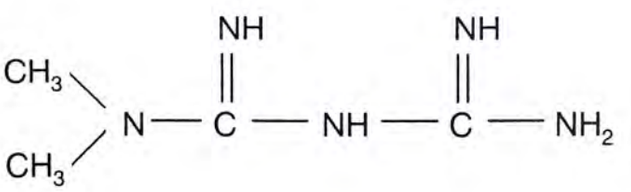


Figure 1.3. The structure of metformin.

Metformin is an effective anti-hyperglycaemic agent, which lowers blood glucose mainly by inhibiting hepatic gluconeogenesis and therefore reducing

hepatic glucose output into the bloodstream (Crofford, 1995). To a lesser extent, metformin can also stimulate both basal and insulin-stimulated glucose uptake in the peripheral tissues, e.g. adipose tissue and skeletal muscle (Ciaraldi *et al.,* 2002; Kumar and Dey, 2002). Lactic acidosis is a well recognized complication of biguanide therapy (Chan *et al.*, 1999).

iii. Thiazolidinediones

Thiazolidinediones is a new class of drug for the treatment of type 2 diabetes. This class of drugs is shown to have an enhancing effect on insulin sensitivity by decreasing hepatic gluconeogenesis and increasing glucose uptake and metabolism in the peripheral tissues, especially skeletal muscles and adipose tissue (Wagstaff and Goa, 2002).

Troglitazone, the first thiazolidinediones available for clinical use, was, however, soon withdrawn from the market because of its hepatic toxicity. Its successors, rosiglitazone and pioglitazone, are found not to contain such toxicity (Isley, 2003). However, weight gain and increased LDL level are the adverse effects of thiazolidinediones treatment (O'Moore-Sullivan and Prins, 2002).

iv. α-Glucosidase inhibitor

α-Glucosidase inhibitor can reduce postprandial glucose absorption from the intestine. Acarbose is the most commonly used α-glucosidase inhibitor clinically. Its structure is similar to the oligosaccharides, a digestion product from starch, and it is a reversible inhibitor of the digestive enzyme α-glucosidase, which is present in the brush border of the small intestine. The drug

acts by competitively inhibiting the enzymes, thereby inhibiting the hydrolysis of oligosaccharides and disaccharides and hence delaying the absorption of glucose and other monosaccharides (Bischoff, 1994).

There are some minor side effects with the use of α-glucosidase inhibitor, e.g. diarrhea, gas, bloating and abdominal/stomach pain. Although hypoglycaemia is rare, it may occur when it is used with other oral hypoglycaemic agent or insulin (Martin and Montgomery, 1996).

v. Insulin injection

Despite the former name of type 2 diabetes as "non-insulin dependent diabetes", about 40% of the type 2 diabetic patients actually require insulin injection to maintain normoglycaemia because their pancreas fail to produce insulin. These patients are mostly in the late-stage and other oral hypoglycaemic agents fail to rescue the situation. Hypoglycaemia is a major side effect of insulin therapy, which may occur before meal and at night (Campbell and White, 2002).

1.5. The use of herbal medicines in diabetes treatment

Before the discovery of insulin and the other drugs for treating diabetes, herbal medicines were the major form of treatment in the world. Even nowadays, in some developing countries and rural areas, these herbs are still the major form of diabetes treatment. These plants traditionally used for diabetes treatment can provide a source for the new anti-diabetic drug development (Bailey and Day, 1989). For example, the use of French lilac (*Galega officinalis*) in traditional diabetic treatment leads to the discovery

of biguanides (Witters, 2001). There are a number of plants traditionally used for anti-diabetic treatment all over the world. However, only a few of them have been verified for their potency on diabetes treatment. Bitter gourd (*Momordica charantia*) has long been used in diabetes treatment in India. Research showed the water extract of bitter gourd could lower the blood glucose level in alloxan-induced diabetic rats (Virdi *et al.*, 2003). Other herbal materials, e.g. Panax Ginseng, onion bulb and garlic cloves, were also shown to have anti-diabetic effect based on systematic scientific researches (Bailey and Day, 1989). These examples demonstrate the potential of natural products to be incorporated into the medical systems as dietary supplements.

In ancient China, although people had no clear concept of what exactly diabetes is, they developed their own system on the knowledge of the human body and they recognized diabetes as "Xiaoke" symptom (消渴症), which is characterized by thirst, polyuria and weight loss. This symptom was described as early as 2000 years ago in "Huang Di Nei Jing" (黃帝內經, The Yellow Emperor's classic of internal medicine) and recognized as the results of inborn defects in the organs or disorder in diet (Ji, 2000). Combinations of herbs or herbal formulae, instead of single herb, are usually prescribed for medicinal use in China and a number of Chinese herbal formulae are used to treat the disorder and ameliorate the symptoms. According to a brief review, Dang-Gui-Bu-Xue-Tang (Dong Quai & Astragalus Combination, 當歸補血湯), Luwei-Dihuang-Fufang (Rehmannia Six Formula, 六味地黃複方), Si-Jun-Zi-Tang (Major Four Herb Combination, 四君子湯) and Yu-Nu-Jian (Rehmannia & Gypsum Combination, 玉女煎) are the common traditional Chinese medicine (TCM) formulae used to treat the "Xiaoke" symptom. Radix Astragali (黃耆), Radix Rehmanniae (生地), Radix

Trichosanthis (天花粉), Rhizoma Coptidis (黃連), Radix Ophiopogonis (麥冬), Rhizoma Anemarrhenae (知母) and Radix Scrophulariae (玄參) are the common ingredients of these formulae (Wu and Ren, 2002). Some of them were studied for their anti-diabetic effects using scientific research methods and have shown anti-hyperglycaemic effects in animal studies or in non-standardized clinical trials (Huang and Xu, 2002; Yang, 2003; Wang *et al.*, 2001). This implies that the traditional use of the Chinese herbs in treating diabetes is not only a folk legend. However, sufficient and concrete evidence on the efficacy and the safety, as well as the mode of action, of these herbs are still lacking. Therefore, in-depth studies on the anti-diabetic activities of these Chinese herbs are much needed.

Two traditional Chinese medicine formulae, namely 托毒生肌顆粒劑 (Formula 1) and 耆味地黃顆粒劑 (Formula 2), were studied for their anti-diabetic effect on type 2 diabetes. The anti-diabetic effects of the formulae, as well as their component herbs were tested because of several reasons:

a) The formulae showed promising effect on healing the ulcer and over 80% of the patients were saved from amputation in a preliminary clinical study (Wong *et al.*, 2001). We would like to explode the possibilities that the formulae and their individual components may possess anti-diabetic activities since glycaemic control of the foot ulcer patients is one of the effective ways to treat the ulcer.

b) Some of the individual components in the formulae were found to be common ingredients of other anti-diabetic formulae, e.g. Radix Astragali and Radix Rehmanniae (Wu and Ren, 2002). It indicates that these herbs may possess anti-diabetic activities. The anti-diabetic effects, as well as the mechanisms mediating such effects, can be confirmed through the investigations.

c) Since the patients taking these formulae for anti-ulcer treatment were also prescribed with modern anti-diabetic medications, and if these formulae have strong hypoglycaemic effect, it may cause undesired hypoglycaemia in the patients.

This project focuses on the effects of formula 1 and its components. The details of formula 1 and its component herbs are discussed in Chapter 2. The effects of formula 2 were studied by Mr. Lau Chun Hong (M.Phil candidate, the Institute of Chinese Medicine, The Chinese University of Hong Kong, another member of the AoE project). The effects of the formulae treatment on diabetic patients were also studied. Since the patients received both formulae treatment, it is impossible to differentiate the actions of each formula in the patients.

1.6. Hypothesis, objectives and design of the project

Based on the fact that formula 1 and formula 2 showed promising effects on healing diabetic foot ulcer and some of the component herbs of formula 1 are used in traditional Chinese medicine for treatment of diabetes, it was hypothesized that formula 1 and its component herbs possess anti-diabetic activities.

The objectives of this project is to test the above hypothesis by conducting the following studies:

1. Authentication of the component herbs in formula 1.
2. Establishment of *in vitro* systems to screen for the herbs with potential anti-diabetic effects.
3. Establishment of an *in vivo* diabetic rat model to confirm the anti-diabetic effects of the herbs screened in objective 2.
4. Investigation on the effects of formula 1 and formula 2 on glucose homeostasis in diabetic patients.

Chapter 2: Preparation and authentication of traditional Chinese medicines

2.1 Introduction

2.1.1 Background information of the formulae

The two Chinese medicine formulae, originally designed for treating diabetic foot ulcer, were 托毒生肌顆粒劑 (formula 1) and 耆味地黃顆粒劑 (formula 2) and they consist of totally 12 individual herbs, including Radix Astragali (黃耆), Radix Rehmanniae (生地), Rhizoma Atractylodis Macrocephalae (白朮), Radix Polygoni Multiflori Preparata (制首烏), Rhizoma Smilacis Chinensis (菝葜), Radix Stephaniae Tetrandra (漢防己), Cortex Moutan (牡丹皮), Fructus Schisandrae Chinensis (五味子), Fructus Corni (山茱萸), Rhizoma Alismatis (澤瀉), Rhizoma Dioscoreae (山藥) and Rhizoma Smilacis Glabrae (伏苓). The two formulae were produced by water extraction of the raw herbal materials, followed by the spray-dry of the water extract. The formulae powders were produced by Hong Kong Institute of Biotechnology Limited (Hong Kong, China).

From a previous study, the two formulae were shown to have successfully rescued over 80% of diabetic foot ulcer cases from amputation. Both formulae contain Radix Astragali and Radix Rehmanniae as the main ingredients. According to the traditional Chinese medicine principles, formula 1 and formula 2 serve different purposes at different stages of diabetic foot ulcer. Formula 1 aims to strengthen the muscle, control swelling, remove the debridement and promote granulation of the wound. Formula 2 aims to promote regeneration and healing of

the wound. Also, formula 2 can alleviate the diabetic symptoms, such as polyuria and thirst (Wong *et al.*, 2001).

In this project, not only the anti-diabetic activity of formula 1, but also that of its component herbs, was investigated. The features of each component herb in formula 1 and the extraction method are described in this chapter.

托毒生肌顆粒劑 (**Formula 1**)		薯味地黃顆粒劑 (**Formula 2**)	
Radix Astragali 黃耆	20g	Radix Astragali 黃耆	20g
Radix Rehmanniae 生地	9g	Radix Rehmanniae 生地	12g
Rhizoma Smilacis Chinensis 菝葜	9g	Fructus Corni 山茱萸	9g
Rhizoma Atractylodis Macrocephalae 白朮	9g	Rhizoma Dioscoreae 山藥	9g
Radix Polygoni Multiflori Preparata 制首烏	9g	Fructus Schisandrae 五味子	6g
Radix Stephaniae Tetrandrae 漢防己	9g	Rhizoma Alismatis 澤瀉	6g
		Rhizoma Smilacis Glabrae 伏苓	6g
		Cortex Moutan 牡丹皮	6g

Table 2.1. The ingredients of formula 1 and formula 2. Each package contains 5g of the formula water extract powder, which was prepared from the raw herbal materials as indicated. The quantities of the raw herbal materials used are given in the table.

2.1.2 Component herbs of formula 1

Formula 1 consists of 6 component herbs as listed in Table 2.1. More details of these herbs are described as follows. For their morphological details, please refer to section 2.3.1.

a) Radix Astragali (黃耆)

Radix Astragali is the root of *Astragalus membranaceus* (Fisch.) Bunge., or *Astragalus mongholicus* Bunge (family: Fabaceae). Radix Astragali is a commonly used medicinal herb in China and its functions and pharmacological actions have been well studied. This herb has immunomodulatory (Chu *et al.*, 1988), anti-aging, diuretic, hypotensive (Chang and But, 1987), anti-bacterial, anti-tumor, skin-reinforcing and tissue generative effects (State Administration of Traditional Chinese Medicine, 1999). The last two effects make this herb beneficial in curing diabetic foot ulcer. Radix Astragali can prevent insulin resistance (Lu *et al.*, 1999). Moreover, Radix Astragali is the major component of many traditional anti-diabetic formulae, such as "Luwei Dihuang Fufang" (Rehmannia Six Formula, 六味地黃複方) and "Fangji Huangqi Tang" (Stephania & Astragalus Combination, 防己黃耆湯). It is believed that this herb has a beneficial effect on the diabetic condition. A recent study showed that one of the chemical compositions in Radix Astragali, named astragalus polysaccharides, possesses hypoglycaemic effect on diabetic rats (State Pharmacopoeia Commission, 2000).

b) Radix Rehmanniae (生地)

Radix Rehmanniae is the rhizome derived from *Rehmannia glutinosa* (Gaertn.) Libosch. (family: Scrophulariaceae). There are two forms of Radix Rehmanniae used as medicinal herbs, the uncured herb (生地) and the cured herb (熟地). The uncured form of herb was used in this project. A report showed that the alcohol extract of the water extract of this herb was able to lower the blood glucose in dogs. The herb also had a hypoglycaemic effect when given to rabbits by oral administration (State Pharmacopoeia Commission, 2000). It is also a main ingredient of the traditional anti-diabetic formula, "Luwei Dihuang Fufang" (Rehmannia Six Formula, 六味地黃複方). Other effects of Radix Rehmanniae include improvement of hemorheology (Kubo *et al.*, 1994), diuretic action, hepatoprotective activity and anti-inflammatory action (State Administration of Traditional Chinese Medicine, 1999).

c) Rhizoma Smilacis Chinensis (菝葜)

Rhizoma Smilacis Chinensis is the dried rhizome of *Smilax china* L. [*S. japonica* (Kunth) A. Gray]. It is usually used to treat rheumatic disease, dysentery and upset stomach (State Pharmacopoeia Commission, 2000). It is also found to have diuretic action and promotional effect on blood circulation (Liu *et al.*, 2002). No report concerning the effects of this herbal material on diabetes has been published.

d) Rhizoma Atractylodis Macrocephalae (白朮)

Rhizoma Atractylodis Macrocephalae is the rhizome of *Atractylodis Macrocephalae* Koidz. (*Atractylodis ovata* Thunb.) (family: Compositae). It is principally used in the treatment of poor appetite, dyspepsia, chronic diarrhea, edema and abnormal fetal movement (State Administration of Traditional Chinese Medicine, 1999). A few studies have been carried out on the anti-diabetic effect of Rhizoma Atractylodis Macrocephalae. Some researches showed that the herb only had a weak hypoglycaemic effect but the results were not conclusive (State Pharmacopoeia Commission, 2000). Other actions of Rhizoma Atractylodis Macrocephalae include diuretic, anticoagulant and tonic effects (Liu *et al.*, 2000).

e) Radix Polygoni Multiflori Preparata (制首烏)

Radix Polygoni Multiflori Preparata is the processed root tuber of *Polygoni Multiflori* Thunb. (family: Polygonaceae). The preparation process is done by mixing the slices of Radix Polygoni Multiflori with black bean juice, followed by stewing, steaming and drying under the sun (State Pharmacopoeia Commission, 2000). This herb is famous for treating premature greying of the hair and easing off the aging process (Chen and Li, 1993). It is also used for promoting heart and vessels functions and lowering blood lipids and cholesterol level (Liu *et al.*, 2000). No report concerning the effects of this herbal material on diabetes has been published.

f) **Radix Stephaniae Tetrandrae (漢防己)**

Radix Stephaniae Tetrandrae is the dried tuber root of *Stephaniae tetrandrae* S. Moore (family: Menispermaceae). This Chinese folk medicine is used as an analgesic, diuretic, hypotensive and tuberculostatic agent (State Administration of Traditional Chinese Medicine, 1999). Radix Stephaniae Tetrandrae had not been investigated alone for its anti-diabetic activity. It is a major component of an anti-obesity formula, Fangji Huangqi Tang (Stephania & Astragalus Combination, 防己黄耆湯). A study found that Radix Stephaniae Tetrandrae possesses anti-hyperglycaemic effect on streptozotocin-diabetic mice and one of its components, fangchinoline, is responsible for such effect (Tsutsumi *et al.*, 1993).

2.2 Objectives

This part aims to conduct the necessary studies prior to the investigation of the anti-diabetic effect of formula 1 as well as its component herbs. The six component herbs of formula 1 were authenticated according to their morphological characteristics and the authenticity of these herbs was further supported by thin layer chromatography (TLC). Then, water extraction of the herbs was performed. Lastly, the sugar contents of formula 1 and the component herb water extracts were determined by high performance liquid chromatography (HPLC) with evaporative light scattering detector (ELSD) system.

2.3 Materials

2.3.1 Raw herbal materials and formula 1 extract

a) Formula 1 (托毒生肌顆粒劑)

Formula 1 was produced by the Hong Kong Institute of Biotechnology Limited (Hong Kong, China), under the Good Manufacturing Practice. Individual herbs were mixed and extracted by water under reflux. Solution was dried using spray-drier and stored in small packages in 5g and each package contains the amount of raw herbal materials as stated in Table 2.1.

b) Traditional Chinese raw herbal materials

Radix Astragali (黃耆), Radix Rehmanniae (生地), Rhizoma Atractylodis Macrocephalae (白朮), Radix Polygoni Multiflori Preparata (制首烏), Rhizoma Smilacis Chinensis (菝葜) and Radix Stephaniae Tetrandra (漢防己), were purchased from Man On Long (Hong Kong, China). All of them were cultivated in China. The six component herbs of formula 1 were firstly authenticated by a morphological expert, Dr. Cao Hui (National Engineering Research Center for Modernization of TCM, Zhuhai, Guangdong, China). Figure 2.1 to Figure 2.6 show the morphology of the component herbs of formula 1. Herbarium voucher specimens were deposited at the museum of the Institute of Chinese Medicine, The Chinese University of Hong Kong, and their voucher specimen numbers are shown in Table 2.2. Their authenticities were further confirmed by TLC method.

Figure 2.1. Morphology of raw materials of Radix Astragali.

Figure 2.2. Morphology of raw materials of Radix Rehmanniae.

Figure 2.3. Morphology of raw materials of Rhizoma Smilacis Chinensis.

Figure 2.4. Morphology of raw materials of Rhizoma Atractylodis Macrocephalae.

Figure 2.5. Morphology of raw materials of Radix Polygoni Multiflori Preparata.

Figure 2.6. Morphology of raw materials of Radix Stephaniae Tetrandrae.

Chinese Herbs	Voucher specimen numbers
Radix Astragali 黃耆	2003-2457
Radix Rehmanniae 生地	2003-2452
Rhizoma Smilacis Chinensis 菝葜	2003-2463
Rhizoma Atractylodis Macrocephalae 白朮	2003-2458
Radix Polygoni Multiflori Preparata 制首烏	2003-2460
Radix Stephaniae Tetrandrae 漢防己	2004-2526

Table 2.2. The voucher specimen numbers of the six component herbs of formula 1.

2.3.2 Thin layer chromatography

a) TLC plate

TLC plates silica gel 60 F_{254} were purchased from Merck (Darmstadt, Germany).

b) Reference compounds

All the reference compounds (Figure 2.7) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

i) **Astragaloside IV** is the reference compound of Radix Astragali (黃耆).

ii) **Catalpol** is the reference compound of Radix Rehmanniae (生地).

iii) **Sarsasapogenin** is the reference compound of Rhizoma Smilacis Chinensis (菝葜).

iv) **Fangchinoline** and **Tetrandrine** are the reference compounds of Radix Stephaniae Tetrandra (漢防己).

v) **Aristolochic acid** is the reference compound of *Aristolochia fangi* (廣防己), the common adulterant of Radix Stephaniae Tetrandra.

c) Reference herbs

Reference herbs of Rhizoma Atractylodis Macrocephalae, Radix Polygoni Multiflori Preparata, Rhizoma Smilacis Chinensis and Radix Stephaniae Tetrandra and they were kindly provided by the Institute of Chinese Medicine, The Chinese University of Hong Kong. These reference herbs had been previously authenticated.

d) Solvents and Sprays

Chloroform, methanol, ethyl acetate, acetonitrile and petroleum ether were purchased from Lab-Scan (Bangkok, Thailand). Acetone, phosphomolybdic acid, vanillin, potassium iodobismuthate and anisaldehyde were purchased from MERCK (Darmstadt, Germany). Sulphuric acid (H_2SO_4) was purchased from BDH Laboratory Supplies (Poole, United Kingdom). Absolute ethanol was purchased from Panreac (Barcelona, Spain). Acetic acid was purchased from Riedel-de Haen (Seelze, Germany). Anisaldehyde TS was freshly prepared from 0.5ml anisaldehyde, 50ml acetic acid and 1ml sulphuric acid (State Pharmacopoeia Commission, 2000).

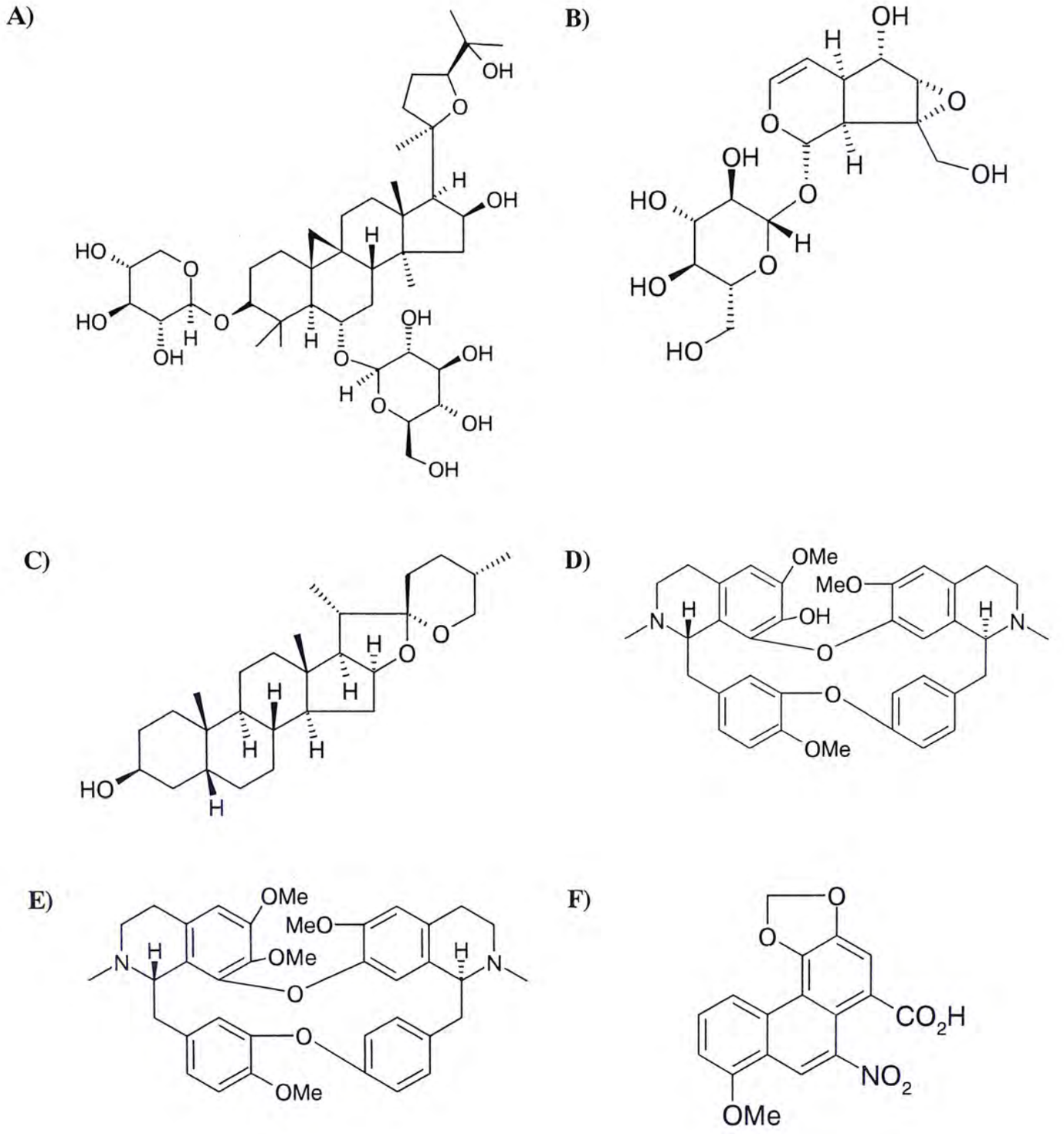


Figure 2.7. The reference compounds used in the TLC analysis of the herbs. (A) Astragaloside IV; (B) Catalpol; (C) Sarsasapogenin; (D) Fangchinoline; (E) Tetrandrine; (F) Aristolochic acid.

2.3.3 High performance liquid chromatography determination of the sugar content of the herbal extracts

a) HPLC column

Prevail Carbohydrate ES 5μ column (250 x 4.6mm) was purchased from Alltech Associates, Inc. (Deerfield, IL, USA). With the use of this column, D-glucose, D-fructose, sucrose and L-rhamnose can be separated effectively.

b) Carbohydrate standards

D-glucose, D-fructose and sucrose content in the herbal extracts were determined by HPLC method using L-Rhamnose (6-deoxy-L-mannose) as the internal standard (Figure 2.8). L-Rhamnose, D-Glucose, D-fructose and sucrose were purchased from Sigma (St. Louis, MO, USA). Around 50mg of L-rhamnose (accurately weighed out) was dissolved in 25ml of water, as the internal standard solution. D-Glucose, D-fructose and sucrose (around 20mg each, accurately weighed out) were mixed and dissolved in 25ml of water, as the stock solution. The stock solution was diluted to different concentrations according to the ratio shown in Table 2.3. One millilitre of the standard solutions from the above dilution was added to six flasks respectively and 1ml of internal standard solution was added to each flask, and water was added to make up the volume of 10ml.

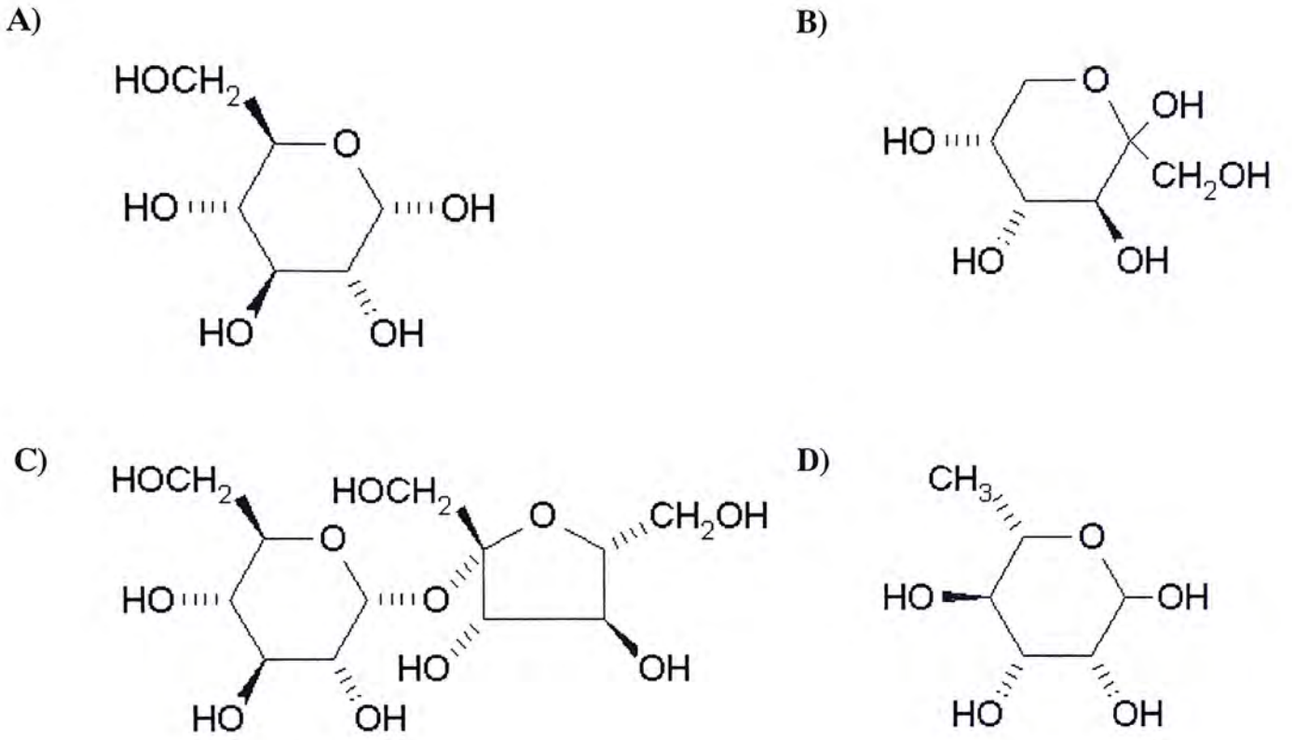


Figure 2.8. The structures of the sugar standards used in the HPLC analysis.
(A) D-Glucose; (B) D-Fructose; (C) Sucrose; (D) L-Rhamnose, the internal standard.

	Volume ratio					
Stock solution	1	2	5	10	15	25
Water	24	23	20	15	10	0

Table 2.3. Dilution of HPLC stock solution.

c) Mobile phase

Acetonitrile was purchased from Lab-Scan (Bangkok, Thailand).

d) Herbal extracts preparation

About 10mg of herbal aqueous extract (accurately weighed out) was dissolved in 10ml of water and 5ml of this solution was mixed with 1ml of L-rhamnose internal standard solution. Lastly, water was added to make up the volume of 10ml. The solution was filtered by Millipore 0.45μm filter to avoid the undissolved residues of the herbal extracts from blocking the HPLC column. The filtrate was then ready for HPLC analysis.

2.4 Methods

2.4.1 Thin layer chromatography of the component herbs

The TLC methods follow that are suggested by the Chinese Pharmacopoeia (State Pharmacopoeia Commission, 2000), except that the method used in Rhizoma Smilacis Chinensis preparation was modified from that suggested by Pharmacopoeia of the People's Republic of China (State Administration of Traditional Chinese Medicine, 1999). The TLC chromatograms of Rhizoma Atractylodis Macrocephalae (白朮) and Radix Polygoni Multiflori Preparata (制首烏) samples were compared to that of the reference herbs because their reference compounds were not commercially available.

a) Preparation of herbal samples

i) Radix Astragali (黃耆)

About 3g of raw herb were grinded into powder and then refluxed with 20ml of methanol for an hour. The solution was filtered and the filtrate was evaporated. The residue was dissolved in 30ml of n-butanol and then extracted twice with 20ml of water to remove the residual water-soluble fractions. Lastly, the n-butanol solution was evaporated and the residue was dissolved in 0.5ml of methanol to make the testing solution.

ii) Radix Rehmanniae (生地)

About 2g of raw herb were grinded into powder and then refluxed with 20ml of methanol for an hour. The solution was filtered and the filtrate was collected. The filtrate volume was reduced to 5ml under reduced pressure using rotary

evaporator (Brinkmann, Westbury, NY, USA) and the concentrate was used as the testing solution.

iii) Rhizoma Smilacis Chinensis (菝葜)

About 5g of raw herb were grinded into powder and then refluxed with 50ml of ethanol for an hour. The solution was filtered and the filtrate was collected, then evaporated to complete dryness using rotary evaporator (Brinkmann, Westbury, NY, USA). The residue was refluxed with 20ml of 10% sulphuric acid for 3 hours. The solution was then extracted twice with chloroform. The chloroform layer was collected and evaporated to complete dryness using rotary evaporator (Brinkmann, Westbury, NY, USA). The residue was dissolved in 1ml of hexane as the test solution. The authentication of Rhizoma Smilacis Chinensis was also made by the reference herb, which was prepared under the same condition as the tested herb.

iv) Rhizoma Atractylodis Macrocephalae (白朮)

About 0.5g of raw herb were grinded into powder and then ultrasonicated with 2ml of n-hexane for 15 minutes. The solution was filtered and the filtrate was collected as testing solution. The authentication of Rhizoma Atractylodis Macrocephalae was made by the reference herb, which was prepared under the same condition.

v) Radix Polygoni Multiflori Preparata (制首烏)

About 0.25g of raw herb were grinded into powder and then refluxed with 50ml of ethanol for an hour. The solution was filtered and the filtrate was

collected. The filtrate volume was reduced to 3ml using rotary evaporator (Brinkmann, Westbury, NY, USA) under reduced pressure. The authentication of Radix Polygoni Multiflori Preparata was made by the reference herb, which was prepared under the same condition.

vi) Radix Stephaniae Tetrandrae (漢防己)

About 1g of raw herb were grinded into powder and dissolved in 6 drops of ammonia, followed by reflux with chloroform for 6 hours. The chloroform layer was retained and the chloroform was evaporated. The residue was dissolved in 2ml of ethanol, as the testing solution. The authentication of Radix Stephaniae Tetrandrae was also made by the reference herb, which was prepared under the same condition.

b) Preparation of reference compounds

Reference compounds were dissolved in methanol to make up to 1mg/ml solutions.

c) Thin layer chromatography

The prepared herbal samples and the reference compound solution were spotted, using capillary tubes, on the baseline about 1cm from the bottom edge of the TLC plates, silica gel 60 F_{254}. Ten milliliters of mobile phase was added to a covered glass chamber. After five minutes, the TLC plate was placed inside the pre-equilibrated chamber to allow the capillary movement of the mobile phase. The plate was removed when the solvent front of the mobile phase reached about 1cm below the top edge of the plate and it was allowed to dry in the air. The TLC profiles were

detected and visualized using different methods. The mobile phases and the detection methods used for different herbs are listed in Table 2.3. The chromatograms of the tested were compared to that of the corresponding reference compound and/or reference herb.

Herbs	Mobile phases (v/v)	Detection methods
Radix Astragali 黃耆	Chloroform / methanol / water (13:7:2)	The TLC plate was sprayed with 10% sulphuric acid in ethanol and then heated at 110°C for 10 minutes and then examined under UV light (365nm).
Radix Rehmanniae 生地	Chloroform / methanol / water (14:6:1)	The TLC plate was sprayed with anisaldehyde TS and then heated at 105°C for 5 minutes until distinct spots were observed.
Rhizoma Smilacis Chinensis 菝葜	Chloroform / ethyl acetate (9:1)	The TLC plate was sprayed with 7% ethanolic phosphomolybdic acid.
Rhizoma Atractylodis Macrocephalae 白朮	Petroleum ether (60-90°C) / ethyl acetate (50:1)	The TLC plate was sprayed with 5% solution of vanillin in sulphuric acid and then heated at 105°C until distinct spots were observed.
Radix Polygoni Multiflori Preparata 制首烏	Acetonitrile / water (25:75)	The TLC plate was examined under UV light (365nm).
Radix Stephaniae Tetrandrae 漢防己	Chloroform / acetone / methanol / ammonia (20:3:2:0.1)	The TLC plate was sprayed with 10% potassium iodobismuthate solution in dilute hydrochloric acid and observed under daylight or UV light (254nm)

Table 2.4. The mobile phases and the detection methods used in the TLC of the component herbs.

2.4.2 Raw herbal materials water extraction

For the water extraction of the raw herbal materials, 500g of the herbal materials were sliced into small pieces and boiled twice in 2L of distilled water for 2 hours under reflux system. The two parts of the aqueous extract were combined and filtered through a cotton cloth. The filtrate was then centrifuged at 15,000 x g for 10 minutes. The supernatant was filtered through Whatman 55mm grade 1 filter paper (Whatman, Brentford, United Kingdom) to remove undissolved residue. The filtrate was frozen and then lyophilized into dry powder by a Thermo Savant MODULYO freeze-dryer (E-C Apparatus Corp., Holbrook, NY, USA) and the powder was stored in desiccator. The extraction product was weighed and the extraction yield was calculated.

2.4.3 High performance liquid chromatography determination of the sugar content of the herbal extracts

The sugar contents of the standard solution and the herbal extract samples were quantified by the use of HPLC-ELSD system. This determination is necessary because all plants contain high amount of carbohydrates, which serve as the structural components as well as energy source and storage. Since studies in this project focuses on glucose metabolism, the sugar content, especially glucose, in the herbal extracts may interfere with the *in vitro* and *in vivo* studies. Therefore, it is necessary to know the amount of sugars in herbal extracts. D-glucose, D-fructose and sucrose content in the herbal extracts were determined because these simple sugars are the most abundant in plants. L-Rhamnose was used as the internal standard because it was not found in the herbal extracts.

Each determination was repeated for three times. The condition of the HPLC-ELSD system is shown in Table 2.5. The peak areas of different sugars were measured by 32 Karat Software version 5.0 (Beckman Coulter Fullerton, CA, USA). The peak area ratios of three sugar standards to the internal standard (e.g. peak area of D-glucose / peak area of L-rhamnose) were plotted against the concentration ratios of sugar standards to the internal standard (e.g. [D-glucose] / [L-rhamnose]) to obtain a calibration curve. The sugar concentration of the herbal extract sample was calculated by the peak area ratio of the corresponding sugar to L-rhamnose using the calibration curve.

HPLC Conditions	
Auto-sampler:	Beckman System Gold 508 Autosampler
Solvent pump:	Beckman System Gold 125 Solvent Module
Stationary phase:	Alltech Prevail Carbohydrate ES 5μ Column (250mm ×4.6mm)
Mobile phase:	Acetonitrile-Water (70:30) (v/v)
Injection volume:	20μl
Flow rate:	1ml/min
Running time:	20min for standards, 30min for testing solutions
ELSD System	
Detector:	Alltech ELSD 2000
Temperature:	80°C
Nitrogen gas flow:	2L/min

Table 2.5. Conditions of HPLC-ELSD system for the determination of the sugar content in the herbal extracts.

2.5 Results

2.5.1 Thin layer chromatography of the component herbs

The six herbal components of formula 1 were authenticated by a morphological expert, Dr. Cao Hui, China, followed by further confirmation using thin layer chromatography (TLC) by comparing to the chromatograms of the reference compounds and/or reference herbs (Figure 2.9 to Figure 2.14). The spots on the chromatograms were identified by a value called retardation factor (R_f)*.

$$* \ R_f \text{ value} = \frac{\text{Distance from baseline to centre of spot}}{\text{Distance from the baseline to the solvent front}}$$

where $0 < R_f < 1$.

The tested herbs, except Radix Astragali, were found to contain compounds with the same R_f value of the corresponding reference compound (marked by the boxes), and/or have similar chromatogram patterns with the corresponding reference herbs. Therefore, the identities of these herbs were proven. The identity of Radix Astragali could not be confirmed by its TLC profile since a number of spots appeared at the position with R_f value of 0.19 (R_f value of Astragaloside IV) (Figure 2.9). The authenticity of Radix Astragali was supported by its morphological study. Since Radix Stephaniae Tetrandrae is commonly adulterated by aristolochic acid-containing *Aristolochia fangi* (Liang *et al.*, 2000), the identity Radix Stephaniae Tetrandrae was further confirmed by the use of aristolochic acid in the TLC system. The tested Radix Stephaniae Tetrandrae sample was found to contain no aristolochic acid and the TLC profile of the tested samples matched that of the reference herb and the reference compounds. Therefore, Radix Stephaniae Tetrandrae was proven to be genuine.

a) Radix Astragali (黃耆)

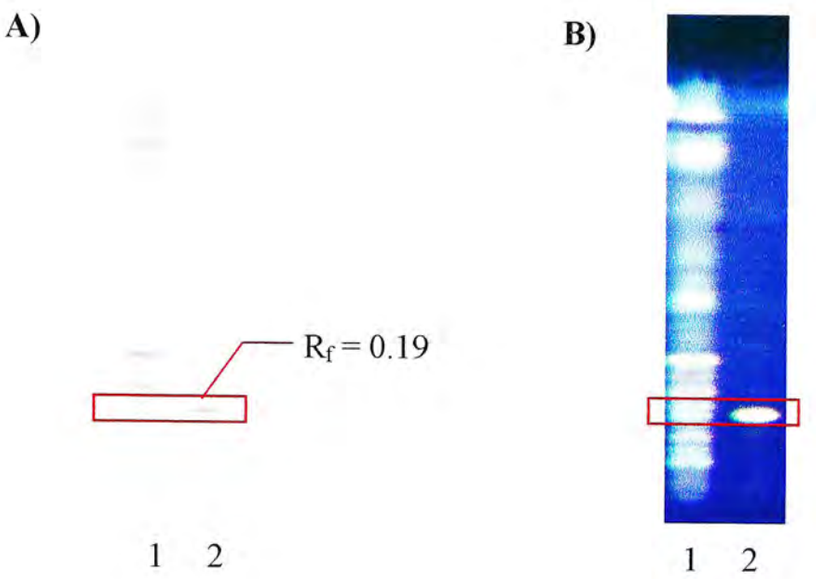


Figure 2.9. The TLC chromatograms of Radix Astragali under (A) visible light and (B) UV light (365nm). Lane 1 - Radix Astragali; Lane 2 - Astragaloside IV.

Stationary phase: Silica gel 60 F_{254}

Mobile phase: Chloroform / methanol / water (13:7:2) (v/v)

Detection method: Sprayed with 10% sulphuric acid in ethanol, heated at 110°C for 10 minutes

b) Radix Rehmanniae (生地)

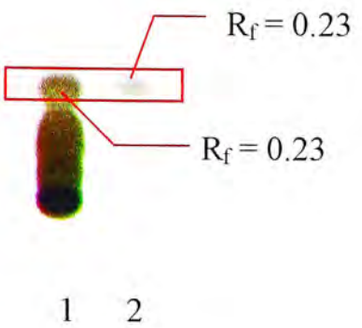


Figure 2.10. The TLC chromatograms of Radix Rehmanniae under visible light.

Lane 1 - Radix Rehmanniae; Lane 2 – Catalpol.

Stationary phase: Silica gel 60 F_{254}

Mobile phase: Chloroform / methanol / water (14:6:1) (v/v)

Detection method: Sprayed with anisaldehyde TS, heated at 105°C for 5 minutes

c) **Rhizoma Smilacis Chinensis (菝葜)**

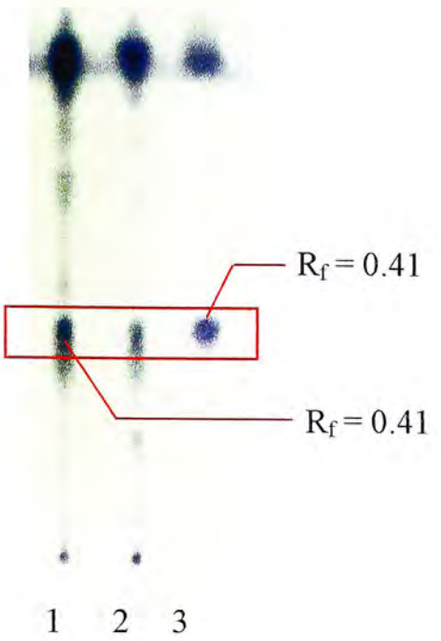


Figure 2.11. The TLC chromatograms of Rhizoma Smilacis Chinensis under visible light. Lane 1 - Rhizoma Smilacis Chinensis; Lane 2 - Rhizoma Smilacis Chinensis reference herb; Lane 3 – Sarsasapogenin.

Stationary phase: Silica gel 60 F_{254}

Mobile phase: Chloroform / ethyl acetate (9:1) (v/v)

Detection method: Sprayed with 7% ethanolic phosphomolybdic acid

d) Rhizoma Atractylodis Macrocephalae (白朮)

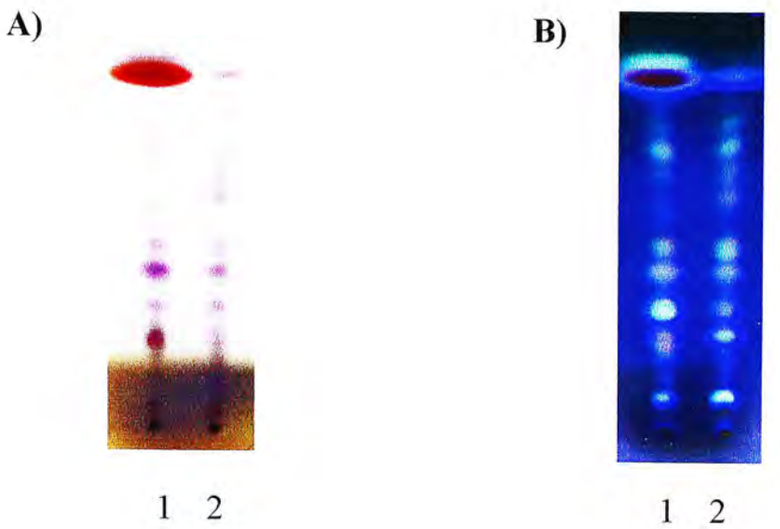


Figure 2.12. The TLC chromatograms of Rhizoma Atractylodis Macrocephalae under (A) visible light and (B) UV light (365nm). Lane 1 - Rhizoma Atractylodis Macrocephalae; Lane 2 - Rhizoma Atractylodis Macrocephalae reference herb.

Stationary phase: Silica gel 60 F_{254}

Mobile phase: Petroleum ether (60-90°C) / ethyl acetate (50:1) (v/v)

Detection method: Sprayed with 5% solution of vanillin in sulphuric acid, heated at 105°C

e) Radix Polygoni Multiflori Preparata (制首烏)

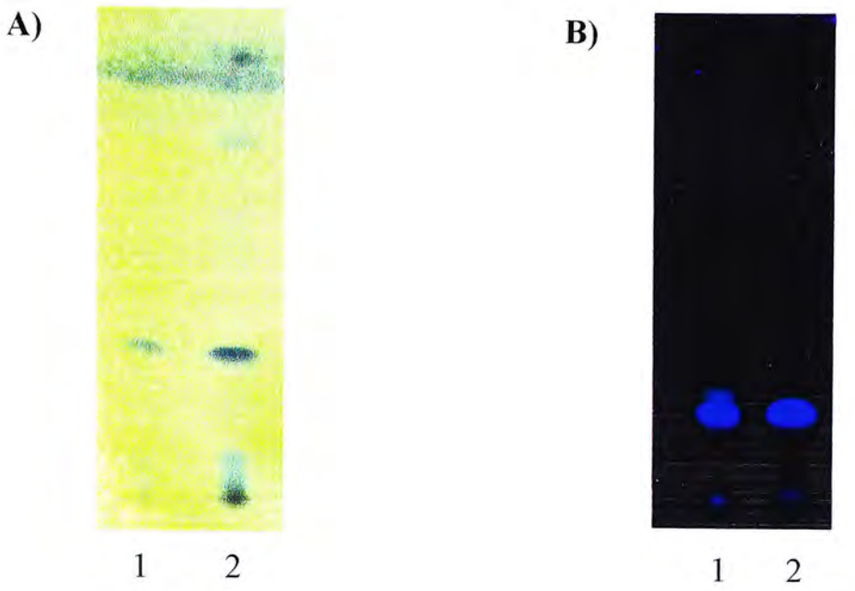


Figure 2.13. The TLC chromatograms of Radix Polygoni Multiflori Preparata under (A) visible light and (B) UV light (365nm). Lane 1 – Radix Polygoni Multiflori Preparata; Lane 2 - Radix Polygoni Multiflori Preparata reference herb.

Stationary phase: Silica gel 60 F_{254}

Mobile phase: Acetonitrile / water (25:75) (v/v)

Detection method: Examination under UV light (365nm)

f) Radix Stephaniae Tetrandrae (漢防己)

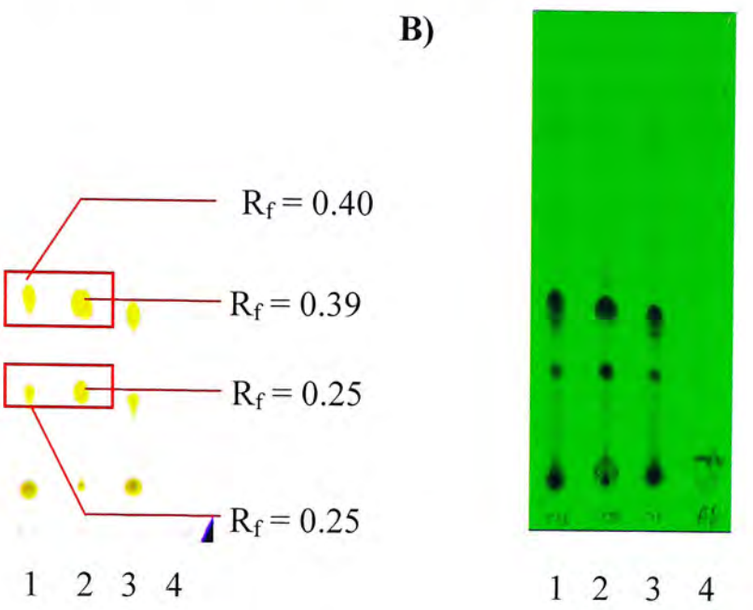


Figure 2.14. The TLC chromatograms of Radix Stephaniae Tetrandrae under (A) visible light and (B) UV light (254nm). Lane 1 –Radix Stephaniae Tetrandrae; Lane 2 – Fangchinoline and Tetrandrine mixture standard; Lane 3 - Radix Stephaniae Tetrandrae reference herb; Lane 4 - Aristolochic acid.

Stationary phase: Silica gel 60 F_{254}

Mobile phase: Chloroform / acetone / methanol / ammonia (20:3:2:0.1) (v/v)

Detection method: Sprayed with 10% potassium iodobismuthate solution in dilute hydrochloric acid

2.5.2 Raw herbal materials water extraction

The component herbs of formula 1 were extracted by water and the water extracts were freeze-dried to produce herbal water extract powder. The yield of extraction was calculated by dividing the weight of the herbal extract product by the weight of the raw herbal materials used for extraction. The percentage yield of extraction of each herb is shown in Table 2.6. The yield ranged from 10.10% (w/w) in Rhizoma Smilacis Chinensis to 48.10% (w/w) in Rhizoma Atractylodis Macrocephalae. Most of the herbs had an extraction yield above 20% (w/w).

Chinese Herbs	Yield of extraction (w/w)
Radix Astragali 黃耆	25.53%
Radix Rehmanniae 生地	47.00%
Rhizoma Smilacis Chinensis 菝葜	10.10%
Rhizoma Atractylodis Macrocephalae 白朮	48.10%
Radix Polygoni Multiflori Preparata 制首烏	21.33%
Radix Stephaniae Tetrandrae 漢防己	26.28%

Table 2.6. Yield of extraction of the six individual herbal components of formula 1. Yield of extraction was calculated by the ratio of the weight of the extraction product to the weight of the raw herbal material used for extraction.

2.5.3 High performance liquid chromatography determination of the sugar content of the herbal extracts

L-Rhamnose, out of a number of sugar standards tested, was chosen as the internal standard for the HPLC analysis because it was not found in the tested herbal extracts and its elution profile does not overlap with the target sugars. The retention time of L-rhamnose, D-fructose, D-glucose and sucrose were about 5min, 6.1min, 7.3min and 9min respectively (Figure 2.15). A complete set of chromatograms is included in Appendix I. The calibration curves for each target sugar were plotted by the peak area ratios of the three sugar standards to the internal standard against the concentration ratios of sugar standards to the internal standard (Figure 2.16). The sucrose content of Radix Astragali extract was out of the range of the standard curve and therefore, the sucrose amount in Radix Astragali extract was determined after five-fold dilution. Since the calibration curve is not a straight line, it does not fit into the normal linear equation. Rather, it fits into the second-order polynomial equation in a single variable x, i.e. $y = ax^2 + bx + c$, where y is the concentration ratio and x is the peak area ratio. The sugar contents of the herbal samples were determined by the corresponding standard curves.

The amounts of D-glucose, D-fructose and sucrose in the herbal extracts are shown in Table 2.7. The results showed that Radix Astragali aqueous extract contains the highest sugar content, 55.52% w/w of its content was the three sugars and most of them were sucrose. Rhizoma Smilacis Chinensis, Radix Stephaniae Tetrandrae and Rhizoma Atractylodis Macrocephalae contain the least amount of sugars, about 12.14% w/w, 14.46% w/w and 13.66 w/w in total,

respectively, and the first two herbal extracts did not contain any sucrose. Generally, all the herbal extracts contained less than 10% w/w of D-glucose and D-fructose.

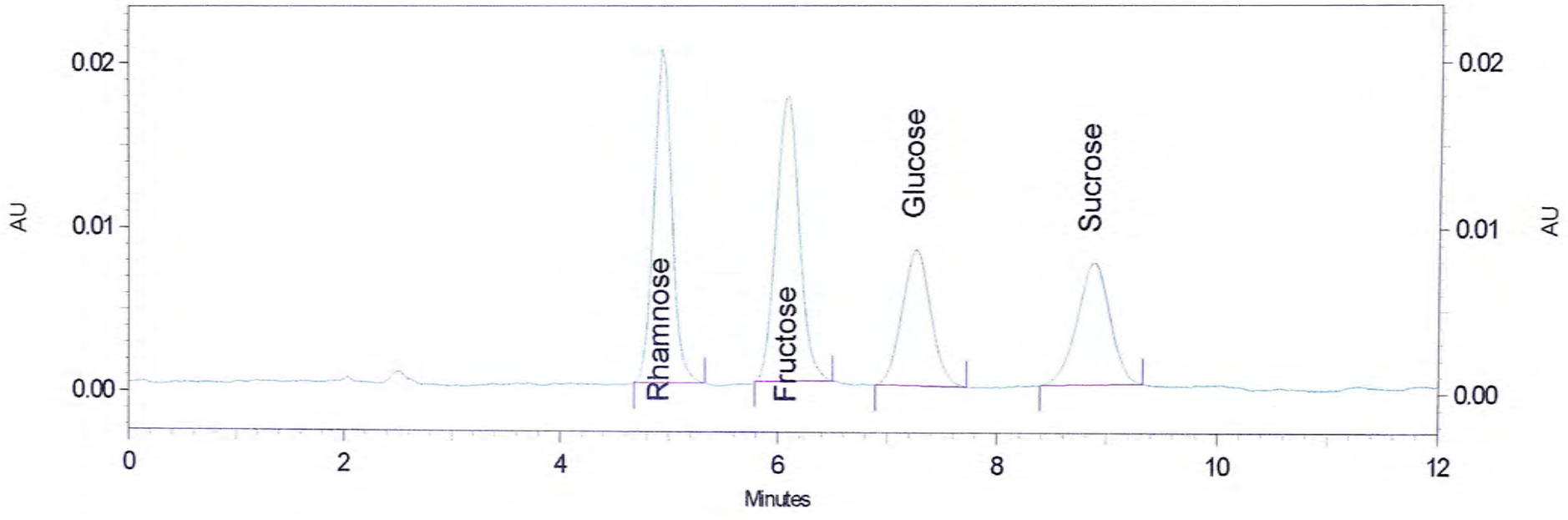


Figure 2.15. The HPLC chromatogram of sugar standards. The retention time of L-rhamnose, D-fructose, D-glucose and sucrose were about 5min, 6.1min, 7.3min and 9min respectively.

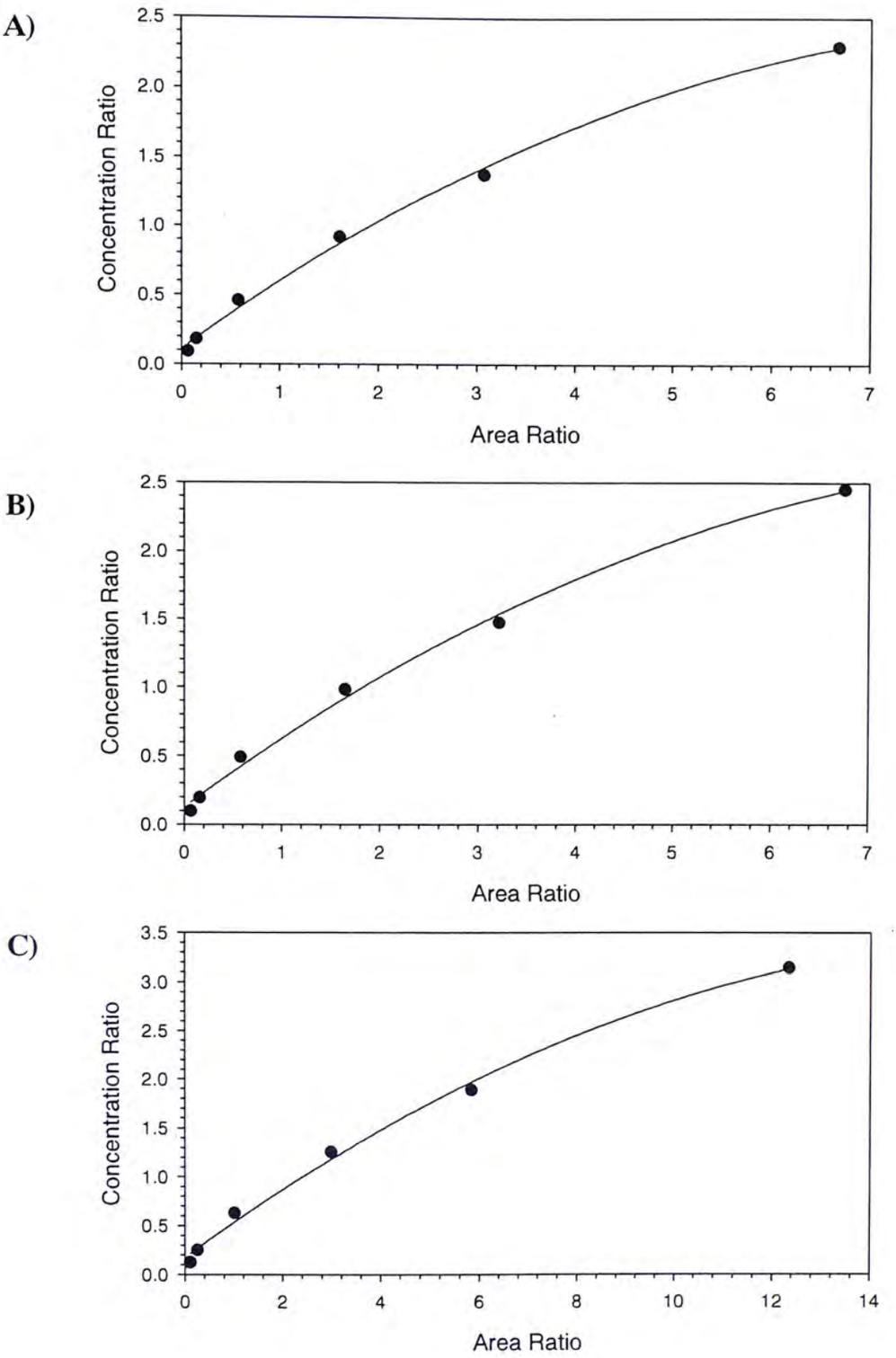


Figure 2.16. Calibration curves of sugar standards.

(a) D-Fructose: $y = -0.0104x^2 + 0.3678x + 0.1746$, $R^2 = 0.9956$;

(b) D-Glucose: $y = -0.0269x^2 + 0.5246x + 0.1270$, $R^2 = 0.9957$;

(c) Sucrose: $y = -0.0284x^2 + 0.5153x + 0.1134$, $R^2 = 0.9967$;

where x-axis is the peak area ratio of the sugars to L-rhamnose and y-axis is the concentration ratio of the sugars to L-rhamnose.

Herbal aqueous extracts	Percentage of total herbal extract weight (w/w)			
	D-Fructose	D-Glucose	Sucrose	Total
Formula 1	6.53	2.06	7.23	19.36
Radix Astragali (黃耆)	5.16	2.08	48.28	55.52
Radix Rehmanniae (生地)	5.79	4.84	7.12	17.75
Rhizoma Smilacis Chinensis (菝葜)	6.83	5.32	0	12.14
Rhizoma Atractylodis Macrocephalae (白朮)	10.60	0.66	2.40	13.66
Radix Polygoni Multiflori Preparata (制首烏)	9.52	8.49	17.99	36.00
Radix Stephaniae Tetrandrae (漢防己)	7.22	7.24	0	14.46

Table 2.7. The content of D-glucose, D-fructose and sucrose in the herbal extracts. The sugar contents of the herbal extracts were calculated by the peak area ratio of the corresponding sugar to L-rhamnose in the herbal extract sample using the calibration curve.

2.6 Discussion

In this chapter, the six herbal components of formula 1 were firstly authenticated according to their morphological characteristics by Dr. Cao Hui, a morphological expert. The identities of these herbs were further confirmed using thin layer chromatography (TLC) by comparing to the reference compounds and/or reference herbs. Although the proof from the TLC analysis of Radix Astragali was not strong, its identity was confirmed by its morphology. The identity of Radix Astragali can be easily distinguished by its texture, taste and cross-section from its counterfeits (Zhang, 2002). Radix Stephaniae Tetrandrae is commonly adulterated by *Aristolochia fangi*. *Aristolochia fangi* contains aristolochic acid, which causes nephropathy (Liang *et al.*, 2000). Therefore, the identity of Radix Stephaniae Tetrandrae was further confirmed by the absence of aristolochic acid in the TLC analysis.

TLC alone is not sufficient to prove the identity of a herb due to the possible overlapping content of the reference compounds present in different herbs. Moreover, chemical variability within the plant materials, as well as the fact that the growth and storage conditions and harvest and post-harvest processes can significantly affect the chemical composition of the herbal species and hinder the confirmation of its botanical identity (Hon *et al.*, 2003). Therefore, TLC data were only used to support the morphological studies. For future studies, both microscopic study and HPLC analysis can be used to provide further evidence for the authentication of the herbs.

After authentication, the herbs were extracted with water and then lyophilized

to produce herbal aqueous extract powder. Water extracts of the herbal materials were employed in this project because these Chinese herbs are traditionally taken through boiling the herbal materials in water, which resembles our present water extraction method. Moreover, formula 1 was made by the water extract of the ingredient herbs. Therefore, it is rational to use the water extracts instead of organic extracts for the subsequent investigation. However, a number of compounds in these herbal materials are non-polar compounds, such as alkaloids. Therefore, the use of water extraction method may inevitably lead to incomplete extraction of the compounds. The percentage yield of the extraction varied greatly among different herbs. The yield of Rhizoma Smilacis Chinensis was particularly lower than the others, which may be due to the fibrous nature of the plant (Ma, 2000).

It is necessary to determine the sugar content in the extract because sugars may interfere with the *in vitro* and *in vivo* assays related to glucose metabolism and homeostasis. Sucrose is broken down into fructose and glucose in digestion. Glucose and fructose are then transported into the bloodstream by sodium-dependent glucose transporter 1 (Sglt1) and glucose transporter 5 (Glut5), respectively. Because D-Glucose, D-fructose and sucrose are the most abundant simple sugars in plants, the amounts of D-glucose, D-fructose and sucrose in the herbal aqueous extracts were determined by HPLC-ELSD system using L-rhamnose as internal standard. The details of the influence of the sugar content in the herbal extracts on the *in vitro* and *in vivo* investigation are discussed in Chapters 3 and 4.

The glucose and fructose content in all extracts are generally low because these

two sugars are more commonly found in the fruits, where all of the component herbs are the root part of the plants. All the extracts generally contain high amount of sugars because the herbs were extracted with water, which can effectively extract polar compounds, such as carbohydrates. Carbohydrates other than glucose, fructose and sucrose present in the extracts may also affect the subsequent investigation of the anti-diabetic effects of the herbs. For example, starch, the most abundant polysaccharide for energy storage in plants, would probably interfere with the *in vivo* experiment because it is digested to glucose for absorption in the intestine. However, the quantification of the starch content in plants is more difficult and is beyond our present technical capacity.

Comparison was made between the extraction yield and the sugar contents between formula 1 and its component herbs and it is shown in Table 2.8. It is found that the extraction yield of formula 1 preparation does not equal to that of the mixture of the component herbs preparation. Moreover, the theoretical contents of glucose and sucrose calculated from the sum of the sugar contents in the component herbs are higher than they should actually be in formula 1. The different extraction methods used in formula 1 preparation (spray-dry) and the component herbs preparation (freeze-dry) may account for such differences. Moreover, the differences in extraction yield and sugar contents may be due to the fact that the component herbs used in formula 1 preparation were different batches to those used in our herbal extract preparation. Different growth, storage, and processing can affect the chemical composition of the component herbs (Hon *et al.*, 2003).

In conclusion, the six component herbs of formula 1 were authenticated by their morphological characteristics and the authenticity of these herbs was further supported by their TLC profiles. Therefore, all the herbs used were proven genuine. Formula 1 and the extraction products of the herbal materials were then used in the subsequent experiments for the study of their anti-diabetic effects *in vitro* (Chapter 3) and *in vivo* (Chapter 4).

Components of formula 1	Amount of the raw herbal materials used in 5g of formula 1 preparation (g)	Estimated amount of water extract after extraction (g)	Amount of sugars in the corresponding amount of water extract present in the formula 1 extract (g)		
			D-Fructose	D-Glucose	Sucrose
Radix Astragali 黃耆	5	1.28	0.066	0.027	0.616
Radix Rehmanniae 生地	2.25	1.06	0.061	0.051	0.075
Rhizoma Smilacis Chinensis 菝葜	2.25	0.23	0.016	0.012	0.000
Rhizoma Atractylodis Macrocephalae 白朮	2.25	1.08	0.115	0.007	0.026
Radix Polygoni Multiflori Preparata 制首烏	2.25	0.48	0.046	0.041	0.086
Radix Stephaniae Tetrandrae 漢防己	2.25	0.59	0.043	0.043	0.000
	Total	4.71	0.346	0.181	0.804

	Amount (g)	Amount of sugars in 5g of formula 1 extract (g)		
		D-Fructose	D-Glucose	Sucrose
Formula 1	5	0.327	0.103	0.362

Table 2.8. Comparison between the extraction yields and the sugar contents between formula 1 and its component herbs. The amounts of water extracts of each component herbs in formula 1 after extraction were estimated by the extraction yield of the component herbs shown in Table 2.6. The amount of sugars of each component herb water extract present in the formula 1 was calculated by the percentage weight of sugars shown in Table 2.7. Comparison was made between the amount of sugars in 5g of formula 1 extract and the theoretical amount of sugars in formula 1 calculated from the sum of the amount of sugars of component herbs water extract.

Chapter 3: The anti-diabetic effects of formula 1 and its component herbs *in vitro*

3.1. Introduction

3.1.1. Glycaemic control in type 2 diabetes

Normal blood glucose regulation is dependent on the feedback loop relationship among the liver, peripheral tissues (muscle and adipose tissue), and pancreatic islet cells (Kahn and Porte, 1996). However, the glucose homeostasis is disrupted in the diabetic state owing to insulin resistance and impaired insulin secretion. Impaired regulation of hepatic glucose production, peripheral insulin resistance and β-cell failure characterize the pathophysiology of type 2 diabetes mellitus (Mahler and Adler, 1999). These abnormalities are responsible for the fasting and postprandial hyperglycaemia in diabetes. The roles of individual malfunctions are discussed in the following sections.

Current treatments of type 2 diabetes are designed to target these abnormalities. As previously stated, there are four main mechanisms for the anti-diabetic drugs to exert their effects to achieve glycaemic control (Heine, 1999) (Figure 1.2):

(1) Stimulation of pancreatic insulin secretion;

(2) Suppression of the hepatic glucose production;

(3) Stimulation of glucose disposal in peripheral tissues;

(4) Retardation of intestinal glucose absorption.

The first three mechanisms obviously target the intrinsic impairments in diabetes. On the other hand, intestinal glucose absorption is not regulated in

normal glucose homeostasis and it shows no abnormality in the diabetic condition. Intestine is targeted in the treatment because type 2 diabetes is characterized by impaired glucose tolerance, causing a postprandial upsurge of blood glucose level (Davis *et al.*, 1994). Theoretically, therefore, drugs that can retard the postprandial glucose upsurge may alleviate the adverse consequence of postprandial hyperglycaemia on the β-cell function and insulin sensitivity (Yki-Jarvinen, 1992). Most of the current and developing drugs aim at the above mechanisms to exert their anti-diabetic effects. More details are given in section 3.1.4.

As the four mechanisms mentioned above are the main modes of action of the anti-diabetic drugs, formula 1 and its component herbs were firstly investigated for their effects on the target tissues *in vitro*. In this part, the herbal extracts were tested for their effects on the intestinal glucose absorption, hepatic glucose production and glucose transport into peripheral tissues (adipocytes and fibroblasts as model) (Figure 3.1). The detailed relationship between type 2 diabetes and the target tissues, as well as the rationale of the *in vitro* assay systems, will be described in the following sections.

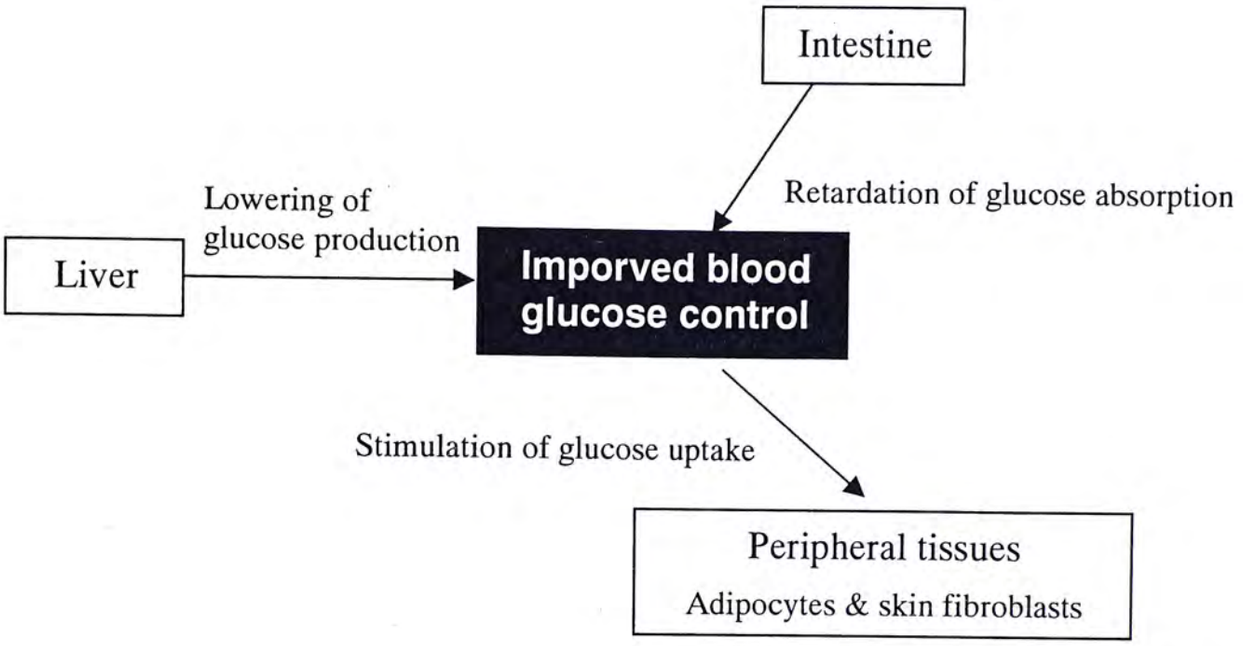


Figure 3.1. Three target mechanisms of the *in vitro* studies of the anti-diabetic effects of herbal extracts in the present study. The herbal extracts were tested for their effects on the *in vitro* systems on liver, intestine and peripheral tissues (adipocytes and fibroblasts as models).

3.1.2. Type 2 diabetes and peripheral tissues

Glucose is the principal energy fuel of all the cells in the body tissues. Cellular glucose uptake also plays a crucial role in maintaining blood glucose homeostasis. Two insulin-responsive peripheral tissues are particularly important in maintaining this balance. They are skeletal muscle and adipose tissue (Bergman, 1997). When the blood glucose level is high, insulin is released from the pancreatic islet and stimulates these tissues to uptake more glucose for respiration or storage. These events promote glucose clearance from the bloodstream and maintain blood glucose at a steady level (Proietto *et al.*, 1983). Nevertheless, insulin resistance in the diabetic condition results in diminished glucose uptake in these tissues (Summers *et al.*, 1999). Apart from insulin-stimulated glucose uptake, researches also found that the basal glucose uptake of these tissues is also lower than normal (Kahn, 2000). Therefore, it is believed that stimulation of both basal and insulin-stimulated glucose uptake of these insulin-responsive tissues or even other insulin non-responsive tissues is beneficial to the amelioration of the diabetic state.

In this part of the experiments, the effects of the Chinese herbs on regulating basal and insulin-stimulated glucose homeostasis in two peripheral tissue models were studied. Fibroblasts were used as the model for peripheral tissue regulating basal glucose homeostasis and it is usually regarded as insulin non-responsive tissue. Adipocytes were used as the model for peripheral tissue regulating insulin-stimulated glucose homeostasis and it is usually regarded as insulin responsive tissue. The effects of the herbal extracts on glucose uptake in these cells were studied.

Fibroblasts provide a convenient system to study the insulin independent glucose transport. Since the skin composes 15% of an individual's total body weight, a reduction of the glucose uptake into skin cells could indeed contribute to the increased glucose level in the blood (Gherzi *et al.*, 1992). The glucose uptake in fibroblasts is mainly mediated via glucose transporter 1 (Glut1), while Glut3 is also present on the plasma membrane (Longo *et al.*, 1992). Research has shown that high glucose level would decrease the glucose uptake as well as the proliferation rate of the skin cells (Spravchikov *et al.*, 2001). In addition, the increase in the glucose uptake in this tissue may also increase the proliferation rate of the cells. This gives implications on the original purpose of formula 1, i.e. to treat the diabetic foot ulcer, because the skin fibroblasts in the ulceration area usually show a lower proliferation rate (Loots *et al.*, 1999).

Hs68 skin fibroblast cell line was chosen as the model because it is a normal skin fibroblast which resembles the skin tissues of the foot ulcer and its fast growth rate allows for rapid screening process. Moreover, skin fibroblast is regarded as an insulin non-responsive tissue and thus allows for the investigation of the effect of the herbal extracts on the basal glucose uptake.

3T3-L1 adipocyte cell line was used as the model for insulin-responsive tissue. The major role of adipose tissue in the body is to act as an energy reserve, storing energy as lipids when the nutrients are abundant while releasing energy during fasting and starvation. The glucose uptake in adipocyte is mediated by both Glut1 and Glut4. Glut1 is responsible for the basal glucose transport while Glut4 is responsible for the insulin-stimulated glucose transport in the adipocytes (Harrison *et al.*, 1992). Under resting state, Glut4 localizes in the intracellular

vesicles. Insulin regulates glucose transport in adipocytes by triggering the translocation of the intracellular Glut4 to the cell surface (Hashiramoto and James, 2000). In adipose tissue from normal subjects, insulin stimulates glucose uptake by approximately two-fold over the basal uptake level. Under the diabetic state, the adipose tissue in the diabetic patients is resistant to the insulin action. Therefore, the insulin-stimulated glucose uptake in the adipocytes is impaired when compared to normal. Moreover, the basal glucose uptake of the adipose tissue is lower than normal (Stolic *et al.*, 2002). Some anti-diabetic drugs, e.g. thiazolidinediones, target insulin-responsive tissues to enhance their glucose transport and improve their insulin sensitivity (Martens *et al.*, 2002). The increase in glucose transport plays a role in the glucose clearance from the bloodstream. Therefore, molecules that can enhance the glucose uptake of adipocytes may have beneficial effects on the diabetic state.

3T3-L1 adipocytes were used as the model of adipocytes because it provides a simple and easy method for drug screening. When 3T3-L1 grows exponentially, the cells maintain fibroblast phenotype. Chemicals, such as 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX) and insulin, can induce the differentiation of these cells into adipocytes. The morphology of adipocytes is different from that of the fibroblasts. Adipocytes show intracellular lipid accumulation (Wu *et al.*, 1998). Fully differentiated 3T3-L1 adipocytes are responsive to insulin that glucose uptake is activated by insulin (Fong *et al.*, 1991).

The effects of the herbal extracts on cellular glucose uptake were measured (Hemati *et al.*, 1997). This assay was conducted by pre-incubating the cells with herbal extracts, followed by the incubation of radioactive glucose analogue, 2-deoxy-D-glucose (2-DG). Then, the amount of the radioactive 2-DG transported into the cell was quantified. Glucose analogue, 2-deoxy-D-glucose, was used instead of glucose because it is not completely metabolized in the cells. 2-deoxy-D-glucose undergoes phosphorylation inside the cells but goes no further in the glycolytic process (Navon *et al.*, 1989).

3.1.3. Type 2 diabetes and liver

The liver plays a central role in regulating glucose homeostasis through the control of glucose production. During fasting state, the liver is responsible for 80% of the glucose release into circulation through glycogenolysis and gluconeogenesis (Stumvoll *et al.*, 1997). The contribution of gluconeogenesis to overall hepatic glucose output increases rapidly with the duration of fasting, as glycogen store is depleted (Landau *et al.*, 1996). In the postprandial state, hepatic glucose output is suppressed by nearly 80% (Selz *et al.*, 2003).

Insulin resistance in type 2 diabetes leads to the inability of insulin to control the activity of gluconeogenic enzymes, and hence, leads to the increase in hepatic glucose output and blood glucose levels (Barthel *et al.*, 2003). Several studies showed that the basal hepatic glucose production in the fasting state is elevated by about 30% in diabetic patients (Staehr *et al.*, 2002). In the postprandial state, suppression of hepatic glucose output is defective and about

50% of excessive glucose is produced by the liver in type 2 diabetic patients (DeFronzo, 1992).

Owing to the importance of liver in glucose homeostasis regulation and the abnormalities of hepatic glucose metabolism found in type 2 diabetes, hepatic glucose production has been focused as the therapeutic target in diabetes. At present, the only approach that targets liver in diabetic treatment is the use of metformin (Stumvoll *et al.*, 1995). Metformin primarily targets at the liver and results in 30% of reduction in basal hepatic output in diabetic patients (Perriello *et al.*, 1994). Minor effects on inhibiting hepatic glucose output have also been reported in sulphonylureas and thiazolidinediones (Staehr *et al.*, 2002).

In this part, the effects of the herbal extracts on gluconeogenesis were studied. Gluconeogenesis is the formation of glucose from non-hexose precursors, such as lactate, glycerol and most of the amino acids, and it takes place mainly in the liver. This process is regulated by antagonistic insulin and glucagon through two rate-limiting enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). The effects of the herbal extracts on PEPCK activity were also studied. The regulation of PEPCK has important impact on the glucose homeostasis. Transgenic mice with over-expression of PEPCK demonstrated diabetes phenotype with hyperglycaemia and impaired glucose tolerance (Valera *et al.*, 1994). Insulin inhibits basal PEPCK gene transcription. However, the PEPCK expression is not suppressed in diabetes (Sasaki *et al.*, 1984). Inhibitors of PEPCK such as metformin and troglitazone would reduce gluconeogenesis and hepatic glucose output, even in the presence of insulin resistance (Lochhead *et al.*, 2001; Yuan *et al.*, 2002; Davies *et al.*, 1999).

The H4IIE rat hepatoma cell line was used as the model to study the effects of the herbal extracts on gluconeogenesis. H4IIE is derived from rat hepatoma (Pitot *et al.*, 1964) with the expression of gluconeogenic enzymes including G6Pase and PEPCK, and the conservation of hormonal responsive properties to insulin (Sasaki *et al.*, 1984). Moreover, H4IIE hepatoma cells are unable to store glycogen and express glucokinase. The glucose produced by gluconeogenesis is not metabolized nor stored as glycogen (Riu *et al.*, 1996). Therefore, H4IIE is commonly used as an *in vitro* model to study gluconeogenesis.

The effects of the herbal extracts on gluconeogenesis were studied by two assays, glucose production assay and PEPCK assay. Glucose produced by the cells was measured by glucose oxidase method (Waltner-Law *et al.*, 2002; Wang *et al.*, 2000). The herbs showing inhibitory effects on the glucose production assay were further studied in the PEPCK assay.

3.1.4. Type 2 diabetes and intestinal glucose absorption

Carbohydrates are the major form of human diet. Glucose absorption from the intestine is the main element contributing to the postprandial glucose input into the bloodstream. In normal subjects, plasma glucose level increases to a peak, usually not exceeding 9mM, in 60-90 minutes after a carbohydrate-rich meal and gradually returns to pre-meal values by 3-4 hours. Through the action of insulin and glucagon, hepatic glycogen storage from glucose is stimulated (Taylor *et al.*, 1996), hepatic glucose release is suppressed (Selz *et al.*, 2003) and glucose disposal into skeletal muscle and adipose tissue is enhanced (Marin *et al.*, 1992) thus restores the plasma glucose level to normal rapidly. However, owing to the insulin resistance and impaired insulin secretion in type 2 diabetes, the above regulatory mechanisms are defected and this results in impaired glucose tolerance and causes postprandial hyperglycaemia (Gerich, 1997).

To control the postprandial blood glucose level, which is vital for the prevention of the complications associated with diabetes, it is important to limit the dietary intake of carbohydrates to prevent the postprandial spike of plasma glucose levels (Creutzfeldt, 1999). However, control by rigid diet restriction is not always possible. Pharmacological interventions that control carbohydrates digestion to release them gradually into the blood would be expected to benefit the patients (Creutzfeldt and Folsch, 1983). Acarbose, an α-glucosidase inhibitor, is used to control glucose absorption by competitively inhibiting the ability of α-amylase and the α-glucosidase enzymes on the small intestinal brush border to break down oligosaccharides and disaccharides into monosaccharides (DeFronzo, 1999).

Beside α-glucosidase inhibitor, there are other possibilities to control the intestinal glucose absorption to prevent the postprandial blood glucose spike, such as inhibition of Na^+-dependent glucose transporter 1 (Sglt1) (Wagman and Nuss, 2001). Sglt1 is responsible for the absorption of glucose from the intestinal lumen into the bloodstream. It co-transports D-glucose and sodium ion across the intestinal brush border membrane, with the stiochiometric ratio of sodium ion to D-glucose 2:1 (Semenza *et al.*, 1984).

To examine the effects of the herbal extracts on the Sglt1, a model called brush border membrane vesicles (BBMV) was used. BBMV are spherical vesicles prepared from the apical membrane microvilli of rabbit intestinal enterocytes (Schmitz *et al.*, 1973. Kessler *et al.*, 1978) and Sglt1 is located on the membrane. The outer surface represents the intestinal lumen and the core of the vesicle represents the intestinal cell. Over 90% of BBMV have the correct orientation, by the interior representing the serosal side (Haase *et al.*, 1978). The advantage of using BBMV in such studies is that Sglt1-mediated glucose transport can be studied without the interference from glucose transporter 2 (Glut2) which locate on the basolateral membrane of the enterocytes (Vedavanam *et al.*, 1999).

To study the effect of the herbal extracts on intestinal glucose absorption *in vitro*, glucose uptake assay on BBMV was performed (Hopfer *et al.*, 1973). In the BBMV glucose uptake experiment, the BBMV were incubated with the herbal extract and the radioactive glucose. Since the final glucose concentration in the BBMV mixture is very low (0.067mM), any trace amount of glucose present in the herbal water extracts would compete with the radioactive glucose

for the Sglt1 and hinder the uptake of the radioactive glucose. Therefore, chloroform extraction on the herbal water extracts was used to remove glucose in the water extracts. However, other polar compounds were inevitably excluded.

3.2. Objectives

In this part, formula 1 and its component herbs were tested for their effects on tissue glucose homeostasis through four *in vitro* systems (Figure 3.1). The objectives of this part of the project are:

1. Testing herbal extracts for their effects on glucose transport in 3T3-L1 mouse adipocytes and Hs68 human skin fibroblasts, which were used as the models of insulin-responsive and insulin non-responsive peripheral tissues, respectively.
2. Testing herbal extracts for their effects on glucose production in H4IIE rat hepatoma cells, which were used as the *in vitro* model of liver gluconeogenesis.
3. Testing herbal extracts for their effects on BBMV glucose uptake, which was used as the *in vitro* model of intestinal glucose absorption.

3.3. Materials

All cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA), all chemicals were purchased from Sigma (St. Louis, MO, USA) and all culture media, serum supplements and antibiotic mixture solution were purchased from Gibco BRL Life Technologies, Inc. (Carlsbad, California, USA) unless otherwise indicated.

3.3.1. Cell lines

a) 3T3-L1 mouse fibroblasts (Chemically induced to adipocytes)

3T3-L1 (ATCC CL-173) is a mouse embryonic fibroblast cell line, which is isolated from Swiss albino mouse and an infinite substrain of 3T3-Swiss albino cell line. Cells can undergo a pre-adipose to adipose-like conversion as they progress from a rapidly dividing state to a confluent and contact inhibited state or by chemical induction.

b) Hs68 human fibroblasts

Hs68 (ATCC CRL-1635) is a human fibroblast cell line. It is one of a series of human foreskin fibroblast lines obtained from normal Caucasian newborn male.

c) H4IIE rat hepatoma cells

H4IIE (ATCC CRL-1548) is a rat hepatoma cell line. The H4IIE hepatoma cell line has been used by many investigators to study insulin action because insulin can act on these cells at picomolar concentrations to induce many effects normally associated with growth factors (Schwartz, 1993).

3.3.2. Cell culture media, buffers, reagents and culture wares

a) Cell culture media

i) Dulbecco's modified Eagle medium (DMEM)

The powder form of DMEM medium supplemented with 4,500mg/L of D-glucose, 4mM of L-glutamine, and 110mg/L of sodium pyruvate and 4mg/L of pyridoxine HCl, was used for the preparation of culture medium. The powder was dissolved in double distilled water and buffered with 3.7g of sodium bicarbonate ($NaHCO_3$) per litre. The pH value of the medium was adjusted to 7.2 to 7.4 by 6M hydrochloric acid (HCl) and the medium was sterilized by filtration through a 0.22μm Millipore filter (Millipore, Billerica, Mass, USA) and stored at 4°C until use.

ii) Dulbecco's modified Eagle's medium (DMEM, without glucose)

The liquid form of no glucose DMEM medium supplemented with 4mM L-glutamine was used for experiment of 3T3-L1 and Hs68 cell lines. It was stored at 4°C until use.

iii) Dulbecco's modified Eagle's medium (DMEM, without glucose and phenol red)

Glucose-free Dulbecco's modified Eagle's medium was purchased from Sigma (St. Louis, MO, USA). It contains no phenol red, glucose, sodium pyruvate and sodium bicarbonate. The preparation and the storage of this medium was the same as the standard Dulbecco's modified Eagle medium. This medium was used in the glucose production assay and PEPCK assay in H4IIE cells.

b) Serum supplements

Fetal bovine serum (FBS) was stored as 50ml aliquots in sterile centrifuge tubes and the aliquots were then kept frozen at –70°C until use.

c) Antibiotic mixture solution

Penicillin-Streptomycin (PS) antibiotic mixture stock solution containing 10000units/ml of penicillin G sodium plus 10000μg/ml of streptomycin sulphate in 0.85% saline was stored as aliquots of 10 ml in sterile centrifuge tubes at -70°C.

d) Complete medium

DMEM (high glucose) medium supplemented with 10% (v/v) FBS and 1% (v/v) PS was used for sub-culturing of all the studied cell lines.

e) Phosphate-buffered saline (PBS)

Stock PBS solution (10x) was prepared by dissolving 80g of sodium chloride (NaCl), 2g of potassium chloride (KCl), 14.4g of dibasic sodium phosphate ($Na_2HPO_4 \bullet 2H_2O$), 2.4g of monobasic potassium phosphate ($KH_2PO_4 \bullet H_2O$) in one litre of double distilled water with pH calibrated to 7.4 by 6M HCl or 2M sodium hydroxide (NaOH). A working solution (1x) was prepared by diluting 100ml of 10x PBS (warm before use) with 900ml of double distilled water and then sterilized by autoclaving at 121°C for 20 minutes. It was stored at 4°C until use.

f) Trypsin-EDTA Solution

Trypsin-EDTA solution (without Ca^{2+} and Mg^{2+}) containing 0.05% (w/v) trypsin and 0.53mM of EDTA-tetrasodium in Hank's balanced salt solution was stored at –70°C as 50 ml aliquots until use.

g) Cell Culture Wares

Glasswares used for sub-culture and experiments, including flasks, multi-well plates and centrifuge tubes, were all purchased from Iwaki (Chiba, Japan).

3.3.3. Chemicals, media and reagents for 3T3-L1 differentiation

a) 3-Isobutyl-1-methylxanthine (IBMX)

IBMX powder was dissolved in 0.5M of potassium hydroxide (KOH) as a stock solution of 50mM. The solution was sterilized by passing through a 0.22μm filter and stored as 1ml aliquots at -20°C.

b) Dexamethasone (DEX)

DEX powder was dissolved in 100% ethanol as a stock of 1mM and stored at -20°C as freezer stock. Working stock was prepared by diluting the freezer stock to 0.1mM in sterile PBS and stored at 4°C.

c) Insulin

Insulin solution (bovine origin) (10mg/ml) was stored at 4°C.

d) Oil red O

Oil red O stock solution was prepared by dissolving excess oil red O powder in isopropanol to constitute a saturated solution. This stock solution was stored under dark. The working solution was prepared by diluting the stock solution with distilled water at the volume ratio of 3:2 and it was allowed to stand at room temperature for 7-10 minutes. The solution was then filtered immediately before use.

e) IBMX-DEX-Insulin cocktail medium for 3T3-L1 differentiation.

IBMX-DEX-Insulin cocktail medium was freshly prepared for 3T3-L1 differentiation. The cocktail medium was prepared by supplementing the complete high-glucose DMEM to the final concentration of 0.5mM of IBMX, 0.5μM of DEX and 5μg/ml of insulin. The chemicals were freshly added to the medium before use.

f) Insulin-supplemented medium

Insulin-supplemented medium was freshly prepared for 3T3-L1 differentiation. The medium was prepared by supplementing the complete high-glucose DMEM to the final concentration of 5μg/ml of insulin.

3.3.4. Chemicals and reagents for 3T3-L1 and Hs68 2-deoxy-D-glucose (2-DG) uptake assay

a) 2-Deoxy-D-[1-^{3}H]glucose

The stock solution (1mCi/ml, 14Ci/mmol) was obtained from Amersham (Little Chalfont, United Kingdom) and stored at 4°C.

b) 2-Deoxy-D-glucose (2-DG)

2-Deoxy-D-glucose solution was prepared by dissolving 0.821g of the powder in 500ml of PBS to the concentration of 10mM and stored at 4°C.

c) Liquid scintillation cocktail

The OptiPhase HiSafe 2 scintillation fluid (PerkinElmer Life sciences, Inc., Boston, MA, USA) was stored in the dark at room temperature.

3.3.5. Chemicals and buffers for H4IIE glucose production assay and phosphoenolpyruvate carboxykinase (PEPCK) assay

Insulin and dexamethasone were used in these assays and they were prepared and stored as previously described.

a) 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt (pCPT-cAMP)

pCPT-cAMP powder was dissolved in distilled water to a concentration of 5mM and stored as 1ml aliquots at 4°C.

b) Glucose production buffer

Glucose production buffer was prepared by DMEM solution (without glucose and phenol red) (see Section 3.3.2 (a)(iii)) supplemented with 5mM of sodium pyruvate and 50mM of sodium lactate.

c) Lysis buffer for PEPCK assay

Lysis buffer contained 20mM of Tris-HCl, 50mM of potassium chloride (KCl), 5mM of magnesium chloride ($MgCl_2$), 2mM of EDTA, 1mM of dithiothreitol (DTT) and 1% polyethylene glycol and the pH was adjusted to 6.6.

d) Assay buffer for PEPCK assay

Assay buffer contained 110mM of imidazole-HCl, 45mM of $NaHCO_3$, 2mM of phosphoenolpyruvate, 3mM of $MgSO_4$, 3mM of manganese chloride ($MnCl_2$), 13mM of sodium fluoride (NaF), 1mM of DTT, 0.5mM of GDP, 0.15mM of NADH and 6 units of malate dehydrogenase, and the pH was adjusted to 6.8.

e) Enzymatic-spectrophotometric glucose oxidase/peroxidase assay kit

Enzymatic-spectrophotometric glucose oxidase/peroxidase assay kit was purchased from Biosystems S.A., Spain. The assay kit includes a bottle of assay reagent, containing 70mmol/L of phosphate, 5mmol/L of phenol, more than 10U/ml of glucose oxidase, more than 1U/ml of peroxidase, 0.4mmol/L of 4-aminoantipyrine, at pH 7.5, and a bottle of glucose/urea/creatinine standard, containing 100mg/dl (5.55mmol/L) of glucose, 50mg/dl of urea, 2mg/dl of creatinine.

3.3.6. Animal, buffers and reagents for preparation and glucose uptake assay of brush border membrane vesicles (BBMV)

a) Animal

White New Zealand rabbits were obtained from The Laboratory Animal Services Centre, The Chinese University of Hong Kong.

b) Buffer 1

Buffer 1 contained 10mM of D-mannitol, 2mM of Tris (USB Corp, Cleveland, OH, USA) and the pH was adjusted to 7.1 by 125mM of HEPES.

c) Buffer 2

Buffer 2 contained 100mM of D-mannitol, 0.1mM of $MgSO_4$, 2mM of Tris and the pH was adjusted to 7.4 by 125mM of HEPES.

d) Buffer 3

Buffer 3 contained 300mM of D-mannitol, 0.1mM of $MgSO_4$, 10mM of Tris and the pH was adjusted to 7.4 by 125mM of HEPES.

e) Stop-wash buffer

The stop-wash buffer contained 200mM of NaCl, 10mM of HEPES-Tris and 250μM of phlorizin (other names: phlorhizin, phloridzin). Phlorizin is a Sglt1 inhibitor used to stop the glucose uptake by the BBMV.

f) D-[2-^{3}H]glucose

The stock solution (1mCi/ml, 15-20Ci/mmol) was obtained from Amersham (Little Chalfont, United Kingdom) and stored at 4°C.

3.3.7. Reagents for bicinchoninic acid (BCA) protein assay

a) Bicinchoninic acid

Bicinchoninic acid was stored at room temperature.

b) Copper (II) sulphate pentahydrate solution

Copper (II) sulphate pentahydrate was prepared as a 0.16M solution and stored at room temperature.

c) Bovine serum albumin (BSA)

BSA standard was prepared as 10mg/ml stock in autoclaved double distilled water and stored as 1ml aliquots at –20°C.

3.4. Methods

3.4.1. Cell culture

3T3-L1 mouse fibroblasts, Hs68 human fibroblasts and H4IIE rat hepatoma cells were cultured in complete high-glucose DMEM (contained 10% (v/v) FBS and 1% (v/v) PS (final concentration: 100units/ml of penicillin G sodium and 100μg/ml of streptomycin)). The cultures were kept at 37°C, 5% CO_2 atmosphere in a humidified condition. The medium was changed twice or three times a week and subcultured when the cell density reached 70%. 3T3-L1 was maintained between 4^{th} and 13^{th} passages for experiments. Hs68 and H4IIE were maintained between 4^{th} and 20^{th} passages for experiments.

For the subculture procedure, medium was first removed and the adherent cells were rinsed once with pre-warmed PBS before mild trypsinization with 1ml (for 75cm^2 culture flask) or 2ml (for 150cm^2 culture flask) of trypsin-EDTA solution at 37°C for 2-3 minutes. Then, 10ml of fresh medium was added to stop trypsinization when all the cells were detached from the culture flask. Cells were then collected by centrifugation at 200 x g for 3 minutes at 25°C. Following re-suspension in fresh medium, appropriate aliquots of cell suspension were aspirated and dispensed into a new culture flask with fresh medium (15ml for 75cm^2 flask or 25ml for 150cm^2 flask).

Long-term storage of cell line was performed by cryo-preservation in liquid nitrogen. Cells were allowed to grow until 80% confluence and trypsinized as described previously. The cell pellet was re-suspended in 1ml (for 75cm^2 culture flask) or 2ml (for 150cm^2 culture flask) of fresh medium. Then,

1ml of cell suspension was dispensed into pre-labelled cryogenic vial with 10% cryoprotectant dimethyl sulphoxide (v/v) (Sigma, St. Louis, MO, USA). The vials were placed in a Nalgene freezing container at –70°C freezer to achieve 1°C/minute cooling rate. After an overnight incubation, the vials were transferred to liquid nitrogen freezer.

3.4.2. Studies on glucose uptake in 3T3-L1 adipocytes and Hs68 fibroblasts

3.4.2.1 Differentiation of 3T3-L1 cells

3T3-L1 fibroblast cells were chemically induced into differentiated adipocytes before glucose uptake experiments as previously described (Hemati *et al.*, 1997), with modifications. Briefly, cells were seeded on the 24-well plates and they were maintained until confluent state. Two days after confluence (set as day 0), the medium was switched to IBMX-DEX-Insulin cocktail medium. On day 3, the medium was switched to the insulin-supplemented medium. After two days, the medium was then switched to normal complete medium and the medium was changed every 2 days. About 90% of the cells showed an adipocytes phenotype of lipid droplets accumulation shown by oil red O staining. The cells were used in experiments on day 9-12 after start of the differentiation.

3.4.2.2 Oil red O staining of the 3T3-L1 cells

The cells were fixed with 2% paraformaldehyde (Sigma, St. Louis, MO, USA). Oil red O working solution was added to the cells and it was allowed to stand at room temperature for 10-15 minutes. The cells were then washed extensively with PBS and the cells were observed under light microscope.

3.4.2.3 2-DG uptake assay of 3T3-L1 adipocytes and Hs68 fibroblasts

The assay was performed as described by Jarvill-Taylor *et al* (2001). Differentiated 3T3-L1 cells seeded in 24-well plates were rinsed once with PBS buffer. Then, the cells were serum-starved with plain high-glucose DMEM (without FBS and PS) for 2 hours. Selected concentrations of TCM water extracts were prepared by dilution in glucose-free DMEM and then filtration through a 0.22μm filter. The cells were incubated with different concentrations of herbal extracts at 37°C for 30 minutes. Negative control experiment was set up by incubating the cells with plain high-glucose DMEM only. Positive control experiment was set up by incubating the cells with 100nM of insulin in plain high-glucose DMEM. The herbal extracts were removed by washing with PBS for 3 times. Then, 300μl Hot/Cold mixture containing of 2μCi/ml 2-deoxy-D-[1-^{3}H]glucose, 0.1mM of 2-DG in glucose-free DMEM were added and the reaction was allowed to proceeded for 15 minutes at 37°C. The reaction was stopped by adding 1ml/well of ice-cold 10mM of 2-DG in PBS solution and each well was washed by the same solution twice. Then, 200μl of 0.5M NaOH was added to lyse the cells. The plates were put on the orbital shaker for 10 minutes to ensure complete lysis and 200μl of 0.5M HCl was then added to neutralize the alkaline. 300μl of the cell lysate was transferred to scintillation vial and 4ml of OptiPhase HiSafe 2 scintillation fluid was added to each sample and radioactivity in aliquots was determined by a Packard Tri-Carb® 2900TR liquid scintillation counter (PerkinElmer Life Science Inc, Boston, MA, USA).

For the glucose uptake assay of Hs68 fibroblasts, cells were seeded in 24-well plates to a confluent state and the glucose uptake assay was performed two days later. The experiment procedure was identical to the 2-DG uptake assay

of 3T3-L1 cell except the followings: Cells were glucose-starved by DMEM without glucose for 1 hour prior to the uptake assay; 100μl of 0.5M NaOH followed by 100μl of 0.5M HCl were added to lyse the cells; and 120μl of the cell lysate was transferred to scintillation vial for radioactivity determination.

In each glucose uptake experiment, a "non-specific binding" set-up was performed. The incubation procedure was the same as the control experiment, with the exception that the addition of radioactive 2-DG was immediately followed by the washing steps. This set-up aimed to determine the non-specific binding of the radioactive 2-DG on the cell membrane surface, not the glucose that was transported into the cells.

The amount of glucose uptake was corrected by protein concentration, which is determined by BCA protein assay described in section 4.4.5. Glucose uptake was calculated as amount of glucose uptake in dpm per milligram of protein. The corrected glucose uptake was subtracted by the non-specific binding value. Results were expressed as the percentage of glucose production of negative control.

3.4.3. Studies on gluconeogenesis in H4IIE hepatoma cells

3.4.3.1 Glucose production assay

The glucose production assay was conducted based on the method described in Waltner-Law *et al* (2002) and Wang *et al* (2000). Briefly, 1.2×10^7 cells were seeded in 90mm dishes. After growing for two days, medium was replaced by medium containing 500nM of DEX and 0.1mM of pCPT-cAMP. It served as the negative control. In treatment groups, herbal extracts were added to the DEX/pCPT-cAMP supplemented medium to the final herbal extract concentration of 0.04, 0.2, 1 or 5 mg/ml. For the positive control group, insulin was added to the DEX/pCPT-cAMP supplemented medium to the final insulin concentration of 10nM. After five-hour incubation, medium was removed and dishes were washed three times with PBS. Then, 2ml of glucose production buffer was added and incubated with the cells for 3 hours. The glucose concentration of the glucose production buffer was determined by an enzymatic-spectrophotometric glucose oxidase/peroxidase assay kit with modification. Briefly, 1ml of glucose production buffer was mixed with 0.3ml glucose assay solution at 37°C for 5 minutes. Absorbance at 500nm was recorded. The protein concentration of each sample was also determined. Dishes were washed with PBS and cells were lysed by 5ml 0.5M of NaOH for 10 minutes. The lysate was neutralized with 5ml 0.5M of HCl. Protein content of samples were determined by BCA assay, as described in section 4.4.5. The amount of glucose production was corrected by protein concentration. Results were expressed as the percentage of glucose production of negative control.

3.4.3.2 PEPCK assay

PEPCK assay was conducted according to previously described methods (Wiese *et al.*, 1991). By the addition of excessive NADH, phosphoenolpyruvate and malate dehydrogenase, PEPCK activity was determined by the rate of NADH consumption using spectrophotometric method (Figure 3.2).

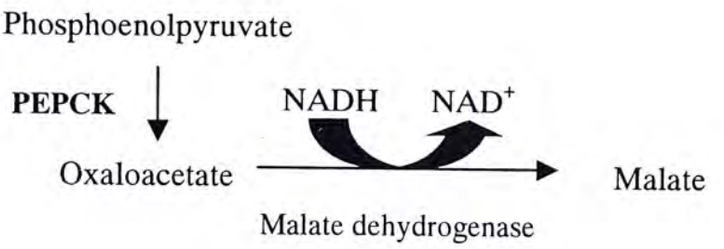


Figure 3.2. The PEPCK activity of the H4IIE hepatoma cells was detected by the rate of NADH consumption. The H4IIE cell lysate was supplied with excessive NADH, phosphoenolpyruvate and malate dehydrogenase. The rate of NADH consumption, therefore, represents the PEPCK activity in the cell lysate.

Briefly, cells were seeded in the 90mm dishes and treated as described in the glucose production assay until after five-hour incubation of DEX/pCPT-cAMP supplemented medium, with or without herbal extract treatment. Negative control experiment and positive control experiment were established in the same condition as in the glucose production assay. After incubation, medium was removed and dishes were washed with PBS twice. Then, 200μl of lysis buffer was added to each dish. Cells were scrapped off, lysed with sonication and centrifuged at 15000 x g for five minutes. Pellets were discarded. Then, 50μl of supernatant was mixed with 500μl PEPCK assay buffer to measure the PEPCK activity.

Absorbance at 340nm of the mixture was monitored for one minute at 15-second intervals. Rate of absorbance change was recorded. Protein content of supernatant was determined by BCA assay. Absorbance change was divided by protein content. PEPCK activity of each sample was expressed as percentage of negative control.

3.4.4 Studies on BBMV glucose uptake

3.4.4.1 Preparation of BBMV

BBMV were prepared based on the Mg^{2+} precipitation method proposed by Schmitz *et al.* (1973) and the modifications of Kessler *et al.* (1978). Rabbits were sacrificed and the small intestine from the pyloric sphincter to ileo-caecal junction was dissected. The mesentery was separated from the intestine. Then, the intestine was placed in 200ml of ice-cold 154mM KCl and processed within one hour. The remaining mesentery and fat were removed and the contents of the intestine were gently squeezed out. The small intestine was cleaned in 200ml of fresh ice-cold 154mM KCl solution. The small intestine was then cut longitudinally and washed thoroughly. Then, the intestine was blotted dry between tissue papers. The small intestine was weighed and then snap-frozen in a plastic bag in liquid nitrogen. The frozen small intestine was stored at -80°C.

BBMV was prepared within one week after the dissection of small intestine. The frozen small intestine was thawed in 200ml of Buffer 1 and was cut into small pieces. The intestine fragments were mechanically vibrated for 1 minute by a Vibro-mixer. Solution was filtered under vacuum to remove connective tissues and muscles and the filtrate was made up to 300ml by Buffer

1. The solution was mixed with 0.61g of $MgCl_2$ by gentle stirring to precipitate the nuclei, mitochondria, basolateral membranes and other cell debris. Then the solution was centrifuged at 4°C, 3800 x g for 15 minutes. The supernatant was saved and centrifuged at 4°C, 43000 x g for 30 minutes. The supernatant was discarded and the pellet was re-suspended in around 5ml of Buffer 2. The solution was centrifuged at 4°C, 43000 x g for 40 minutes. The supernatant was discarded and the pallet was re-suspended in 1ml of Buffer 3. The pelleted volume was measured and the volume was doubled by the addition of Buffer 3. The solution was passed 5 times trough a 25 gauge needle to allow the formation of the BBMV. The solution was divided into 0.26ml aliquots and snap-frozen in liquid nitrogen.

3.4.4.2 Preparation of the chloroform extract of the herbal water extract

The chloroform extract of the water extract of the TCM herbal materials was used to perform the glucose uptake assay of BBMV because the trace amount of glucose in the herbal water extract interfere with the uptake reaction by the BBMV. The chloroform extraction was performed to remove the glucose content in the water extract.

To obtain the chloroform extract of the water extract of the herbal materials, dried herbal aqueous extract was dissolved in distilled water at 200mg/ml. Equal volume of chloroform was added and mixed vigorously with the solution. The mixture was centrifuged at 1000 x g until a clear chloroform layer was separated from the water layer. The lower layer (chloroform) was collected with a pre-weighted glass tube. Extraction was repeated twice as described. The chloroform collections were combined and the chloroform was

completely dried by nitrogen evaporator at 40°C. Weight of dried extract was recorded and the extraction yield was calculated. The chloroform extract was reconstituted to selected concentration by adding appropriate amount of distilled water with sonication.

3.4.4.3 Glucose uptake assay of BBMV

BBMV glucose uptake assay was performed based on the rapid filtration technique (Hopfer *et al.*, 1973). D-glucose was used in the glucose uptake experiment instead of glucose analogs, e.g. 2-deoxy-D-glucose, because the BBMV do not contain glycolytic enzymes inside the vesicles. Therefore, the D-glucose transported into the vesicles would not be metabolized.

Before making use of each batch of prepared BBMV, a time profile glucose uptake assay was carried out as a quality control experiment. One aliquot (0.26ml) of frozen BBMV was re-suspended in 0.44ml of Buffer 3. To start the reaction, 20μl of BBMV was mixed with 40μl of radioactive glucose solution containing 0.1mM of D-glucose, 100mM of sodium thiocyanate (NaSCN), 100mM of mannitol, 10mM of HEPES-Tris, pH 7.4, 10μCi/ml of D-[2-^{3}H]glucose. The reaction was performed at room temperature. In order to obtain the time profile of glucose uptake into BBMV, glucose uptake was stopped after certain incubation times (0, 10, 20, 40, 60, 120, 300, 3600 seconds) by addition of 1ml of ice-cold stop-wash buffer to the incubated mixture, followed by rapid filtration through a pre-wetted 0.45μm nitrocellulose filter (Millipore, Billerica, MA, USA). The filter was washed 5 times with 1ml of ice-cold stop-wash buffer. The filters, which retained the BBMV, were each placed in a scintillation vial

together with 3.5ml of OptiPhase HiSafe 2 scintillation fluid. Radioactivity in aliquots was determined by a Packard Tri-Carb® 2900TR liquid scintillation counter (PerkinElmer Life Science Inc, Boston, MA, USA). The amount of radioactive glucose influx was normalized by the vesicles protein concentration, which was determined by BCA protein assay described in section 4.4.5. The batch of BBMV was functional if the glucose uptake increases sharply with time to a peak at around 10-40 seconds, followed by a gentle decrease of the radioactivity to 3600 seconds.

To screen the effects of the herbal extracts, the glucose uptake of BBMV at fixed time point was performed. One aliquot (0.26ml) of frozen BBMV was re-suspended in 0.44ml of Buffer 3. BBMV (20μl) were mixed with 30μl of the prepared herbal extract solution at appropriate concentration and 10μl of radioactive glucose solution (40μCi/ml of D-[2-^{3}H]-glucose, 0.4mM of glucose, 400mM of NaSCN, 400mM of mannitol, 40mM of HEPES-Tris, pH 7.4). The reaction was performed at room temperature. The glucose uptake reaction was allowed to proceed for 20 seconds (the peak of the glucose uptake). The reaction was stopped and the BBMV was washed as mentioned above. The filters, which retained the BBMV, were subjected to radioactivity counting. The amount of radioactive glucose influx was normalized by the vesicles protein concentration, which was determined by BCA protein assay.

In each experiment, a control and a "non-specific binding" reaction were performed. The control experiment was carried out by incubating the BBMV with distilled water instead of the herbal extract solution. It indicated the

normal glucose uptake reaction without the influence of the herbal extract. The "non-specific binding" reaction was performed under the same condition as the control experiment but ice-cold stop-wash buffer was added prior to the addition of radioactive glucose in order to determine the non-specific binding of the radioactive glucose on the BBMV surface membrane.

Glucose uptake was expressed as amount of glucose uptake (pmol) per milligram of protein. The value of non-specific binding was subtracted from all samples for the correction of non-specific binding. To compare the effect of the herbal extract on the glucose absorption, the glucose uptake of the treatment group was expressed as percentage control.

3.4.5 BCA (Bicinchoninic acid) protein assay

BCA protein assay was performed by following previous protocol (Smith *et al.*, 1985). Briefly, prior to the assay, the assay reagent was freshly prepared by mixing the assay bicinchoninic acid and copper (II) sulphate pentahydrate solution in a ratio of 50:1. . Then, 30μl of each BSA (bovine serum albumin) standard (0-1.25 mg/ml) and 30μl of sample solution were added to 200μl of the assay reagent in 96-well plate wells. Then, the plates were incubated at 37°C for 30 minutes and cooled to room temperature after incubation. The absorbance was measured at 540nm by the Bio-rad model 3550 microplate reader (Bio-rad, Herculus, CA, USA). The protein concentration of the sample was calculated by the standard curve obtained from the BSA standard.

3.4.6 Statistical analysis

For the statistical analysis, data were entered into and analyzed by using SPSS for Windows (version 11.5, SPSS Inc, Chicago, IL, USA). Mann-Whitney tests were used for the comparisons in all experiments between control and each herbal extract treated group, including the glucose uptake in 3T3-L1 adipocytes and Hs68 fibroblasts, the glucose production in H4IIE hepatoma cells and the glucose uptake in BBMV. Statistical tests were two-sided, with a significant level of 0.05.

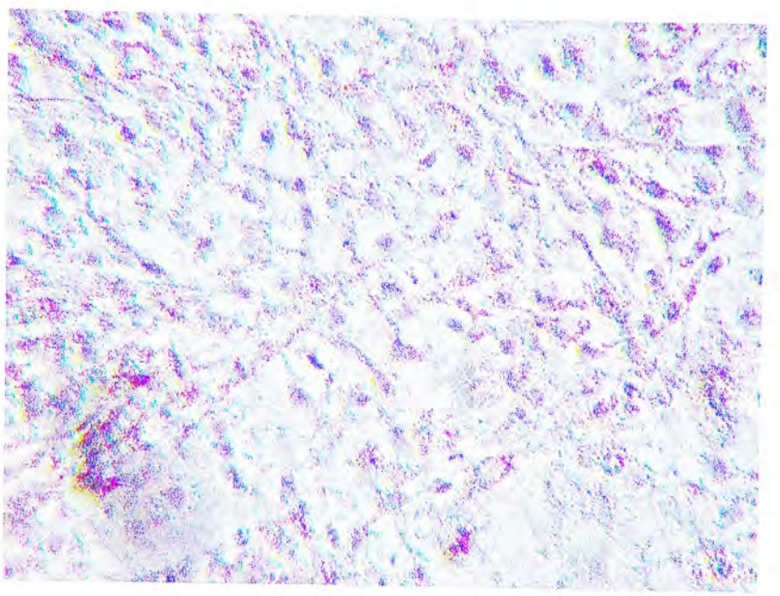
3.5. Results

3.5.1. Glucose uptake assay in 3T3-L1 adipocytes and Hs68 fibroblasts

To confirm the differentiation efficiency of 3T3-L1 adipocytes, the differentiated 3T3-L1 adipocytes were stained by oil red O (Figure 3.3). Oil red O can stain the neutral lipid in red. Cells without chemical induction showed no differentiation into adipocytes and no lipid accumulation. The cells remained typical spindle-shaped 3T3-L1 fibroblast morphology (Figure 3.3A). Cells with chemical induction showed lipid droplets accumulation and showed typical round-shaped 3T3-L1 adipocytes morphology (Figure 3.3B).

Before screening the effects of the herbal extracts, a set of glucose uptake control experiment was carried out in the Hs68 fibroblasts. The time profile of the glucose uptake was obtained to ensure that the time selected (15 minutes) for radioactive 2-DG uptake was within the linear range of the uptake reaction The results showed that the glucose uptake was linearly increasing with time within 30 minutes (Figure 3.4)., which proved that the time selected was appropriate for the screening process. The glucose uptake condition of 3T3-L1 adipocytes was referred to previously established information (Jarvill-Taylor *et al.*, 2001).

A)



B)

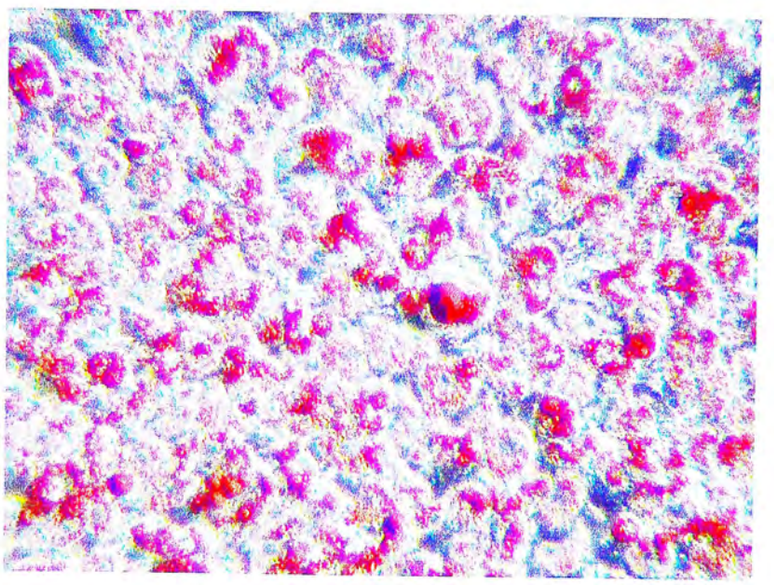


Figure 3.3. Oil red O staining of 3T3-L1 cells (A) before chemical induction and (B) after chemical induction. Cells were fixed with 2% paraformaldehyde and oil red O working solution was added. The cells were allowed to stand at room temperature for 10-15min and then washed extensively with PBS. The stained cells were observed under light microscope.

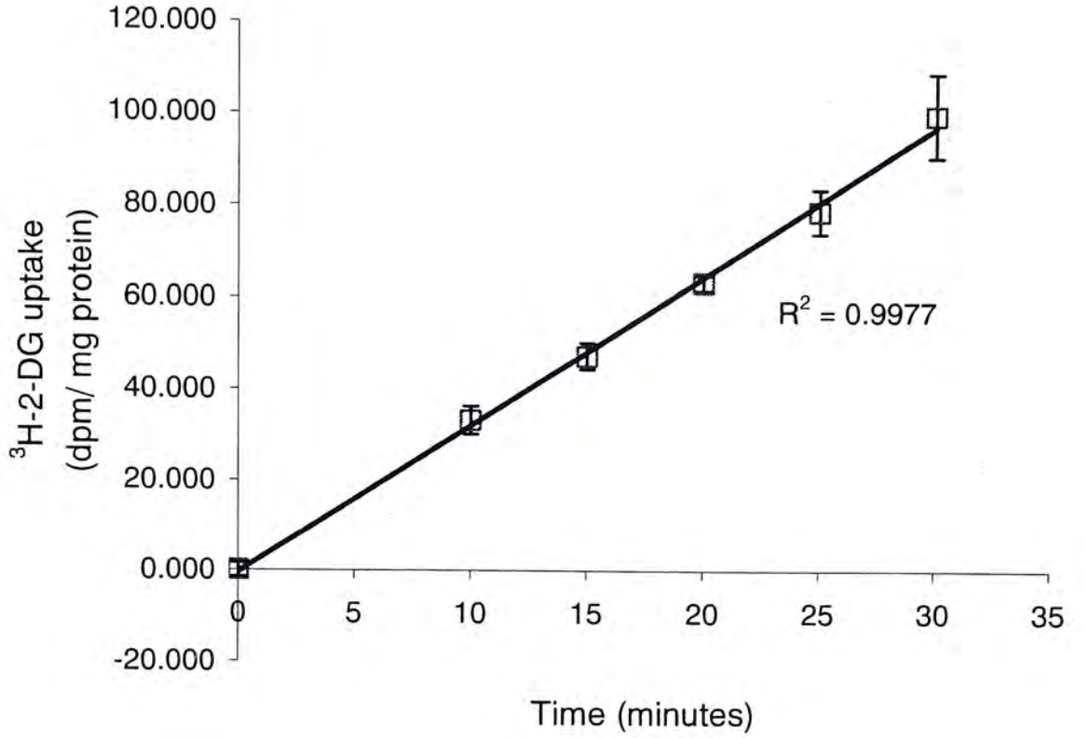


Figure 3.4. The time profile of glucose uptake in Hs68 fibroblasts. Cells were glucose-starved for an hour, followed by the incubation in DMEM containing 2μCi/ml of 2-deoxy-D-[1-^{3}H]glucose and 0.1mM of 2-deoxy-D-glucose. The uptake reaction was stopped at different time points by the addition of ice-cold 10mM of 2-DG solution. The amount of radioactive glucose influx was normalized by cell protein concentration. Data are expressed as mean ± SEM (n = 6).

Formula 1 stimulated glucose uptake of 3T3-L1 and the uptake activity reached its maximum at 1mg/ml extract concentration, increasing the uptake by 64% ($p<0.05$) over the control (Figure 3.5A). Similarly, formula 1 also stimulated glucose uptake in Hs68 fibroblasts (Figure 3.5B). The glucose uptake was increased by about 40%-60% ($p<0.05$).

The 6 component herbs in formula 1, including Radix Astragali (黃耆), Radix Rehmanniae (生地), Rhizoma Smilacis Chinensis (菝葜), Rhizoma Atractylodis Macrocephalae (白朮), Radix Polygoni Multiflori Preparata (制首烏) and Radix Stephaniae Tetrandra (漢防己), were also tested for their effects on the glucose uptake in 3T3-L1 adipocytes and Hs68 fibroblasts (Figure 3.6).

Although Radix Astragali and Radix Rehmanniae showed stimulation in glucose uptake in both cell lines, the trends in the two types of cells were different. In 3T3-L1 adipocytes, Radix Astragali increased the glucose uptake at lower dosage, giving a maximum of 83% of stimulation at 0.1mg/ml ($p<0.05$), but showed inhibition at 10mg/ml. In Hs68 fibroblasts, this herb gave a different trend, showing a dose-dependent increase in the uptake and reaching maximum by increasing the uptake by 40% ($p<0.05$). Radix Rehmanniae exhibited a 58%-68% of stimulation at lower dosage (0.01mg/ml and 0.1mg/ml) in 3T3-L1 adipocytes. However, it did not show modulatory effect on glucose uptake at high concentration (1mg/ml and 10mg/ml). Radix Rehmanniae also exerted a dose-dependent stimulation effect on the Hs68 fibroblast glucose uptake and the uptake reached the maximum at 10mg/ml by increasing the uptake by 31%.

Rhizoma Atractylodis Macrocephalae showed stimulation in glucose uptake in 3T3-L1 adipocytes in lower dosage range (0.01mg/ml-1mg/ml), giving a maximum of 43% increase at 0.1mg/ml ($p<0.05$). However, it only gave minor stimulatory effect by 26.6% on the glucose uptake in the Hs68 fibroblasts at 0.01mg/ml ($p<0.05$).

Radix Polygoni Multiflori Preparata and Rhizoma Smilacis Chinensis stimulated glucose uptake in both cell lines at lower dosage while inhibition was observed at higher dosage. For Radix Polygoni Multiflori Preparata, the stimulation in 3T3-L1 adipocytes reached its maximum at 0.01mg/ml by increasing the uptake by 55% ($p<0.05$) while such stimulation was observed in Hs68 fibroblasts at 1mg/ml by increasing the uptake by 43% ($p<0.05$). Inhibition was observed at 10mg/ml in both cell lines. For Rhizoma Smilacis Chinensis, 83% of stimulation of glucose uptake in 3T3-L1 adipocytes was observed at 0.1mg/ml ($p<0.05$) while such maximal stimulation was observed in Hs68 fibroblasts at 0.01mg/ml by increasing the uptake by 43% ($p<0.05$). Inhibition was observed at 10mg/ml in both cell lines, which complete inhibition on the glucose uptake was observed in Hs68 fibroblasts.

The effect of Radix Stephaniae Tetrandra was screened over a lower concentration range than the other herbs, from 0.1μg/ml to 100μg/ml, because it showed cell death at higher dosages (1mg/ml and 10mg/ml) (Data not shown). It showed no stimulatory effect on the glucose uptake in either cell lines , but an inhibitory effect of 37% to 54% on glucose uptake was observed in Hs68 fibroblasts ($p<0.05$).

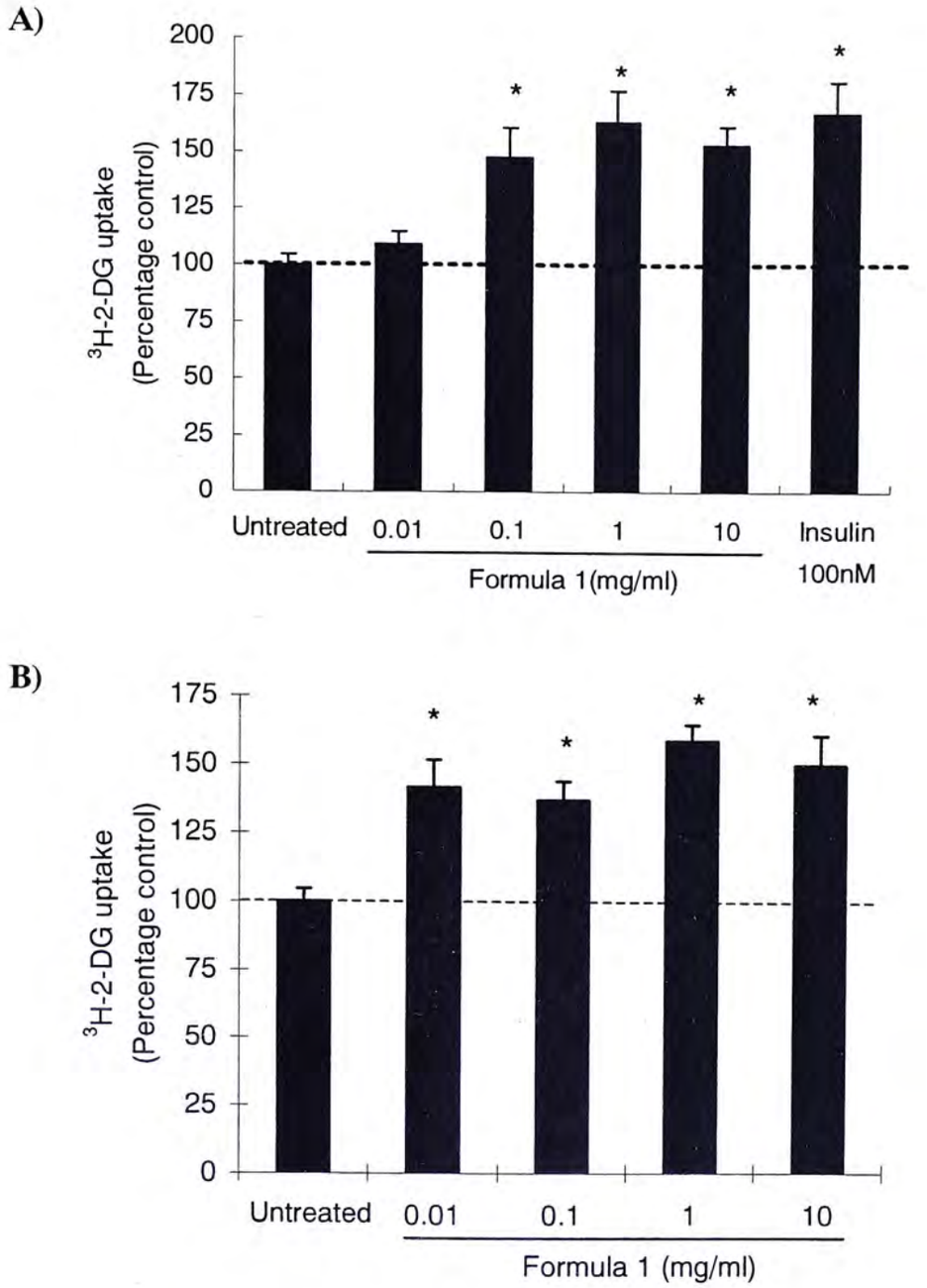


Figure 3.5. Effects of formula 1 on 2-DG uptake in (A) 3T3-L1 adipocytes and (B) Hs68 fibroblasts. 3T3-L1 adipocytes and Hs68 fibroblasts were incubated for 30min without treatment, or with formula 1, or with insulin (100nM, in 3T3-L1 only, serves positive control), followed by the addition of 2-deoxy-D-[^{3}H]glucose for glucose uptake assay. The uptake reaction was stopped at 15min incubation by the addition of ice-cold 10mM of 2-DG solution. The amount of radioactive glucose influx was normalized by cell protein. The glucose uptake values of the herbal extract treated group were expressed as the percentage of the untreated control group. Data are expressed as mean + SEM (n = 30). * Significant difference between untreated control and formula 1 treated groups ($p < 0.05$).

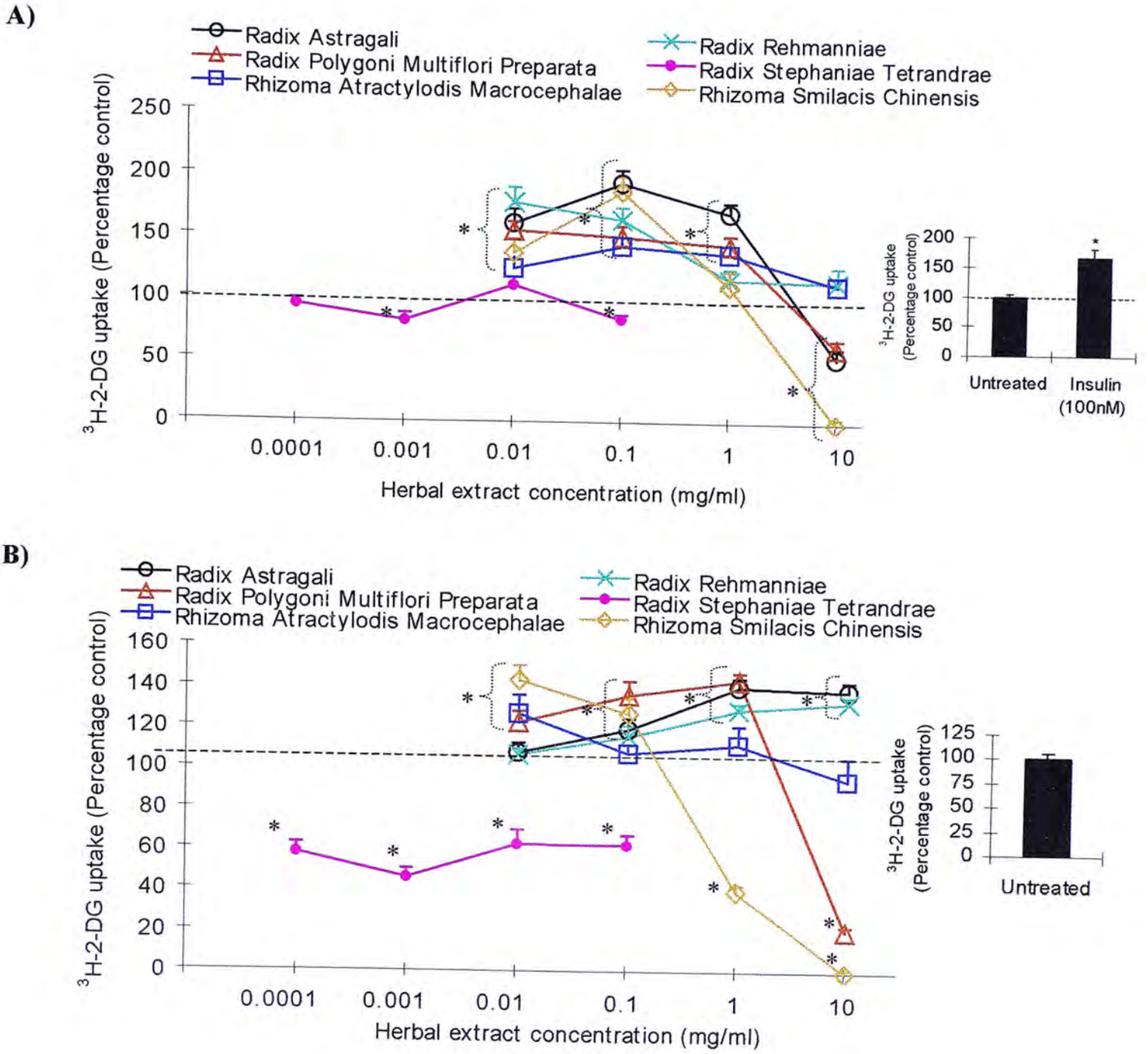


Figure 3.6. Effects of component herbs of formula 1 on 2-DG uptake in (A) 3T3-L1 adipocytes and (B) Hs68 fibroblasts. 3T3-L1 adipocytes and Hs68 fibroblasts were incubated for 30min, with or without herbal extract treatment, or with insulin (100nM, in 3T3-L1 only, positive control (see inset)), followed by the addition of 2-deoxy-D-[^{3}H]glucose for glucose uptake assay. The uptake reaction was stopped at 15min incubation by the addition of ice-cold 10mM of 2-DG solution. The amount of radioactive glucose influx was normalized by cell protein. The glucose uptake values of the herbal extract treated group were expressed as the percentage of the untreated control group. Data are expressed as mean + SEM (n = 18 - 24). * Significant difference between untreated control and herbal extract or insulin treated groups ($p < 0.05$).

3.5.2. Glucose production and PEPCK assay in H4IIE hepatoma cells

Formula 1 and its components were tested for their effects on gluconeogenesis in the H4IIE hepatoma cells using glucose production assay. The limitation of this assay is that a large sample size could not be handled at the same time. Therefore, the test was only repeated for 3 times at each condition with sample size equaling to 1 in each experiment. It was considered undesirable to perform statistical analysis with such a small sample size. Hence, those herbs with comparable effects to insulin treatment (positive control) on inhibition of glucose production were regarded as active herbs. Only the results of these active herbs are presented in Figure 3.7. The results of the other herbs were not promising and their results are shown in Table 3.1. The glucose production assay of Radix Stephaniae Tetrandra at 5mg/ml was not performed because cytotoxicity was observed at this dosage.

From the glucose production assay, only Rhizoma Smilacis Chinensis (菝葜) and Radix Polygoni Multiflori Preparata (制首烏) showed comparable inhibitory effect to insulin at high herbal extract dosages (Figure 3.7). Insulin treatment (10nM) showed 74% of inhibition in the glucose production assay. The glucose production in Rhizoma Smilacis Chinensis treatment group was inhibited by 77.6% and 81.9% at 1mg/ml and 5mg/ml respectively. Radix Polygoni Multiflori Preparata only showed 76.7% of inhibition at 5mg/ml when compared to the untreated group (negative control). However, it showed slight activation of 27.3% at 1mg/ml. Lower dosages of these two herbs did not showed remarkable effect on the glucose production.

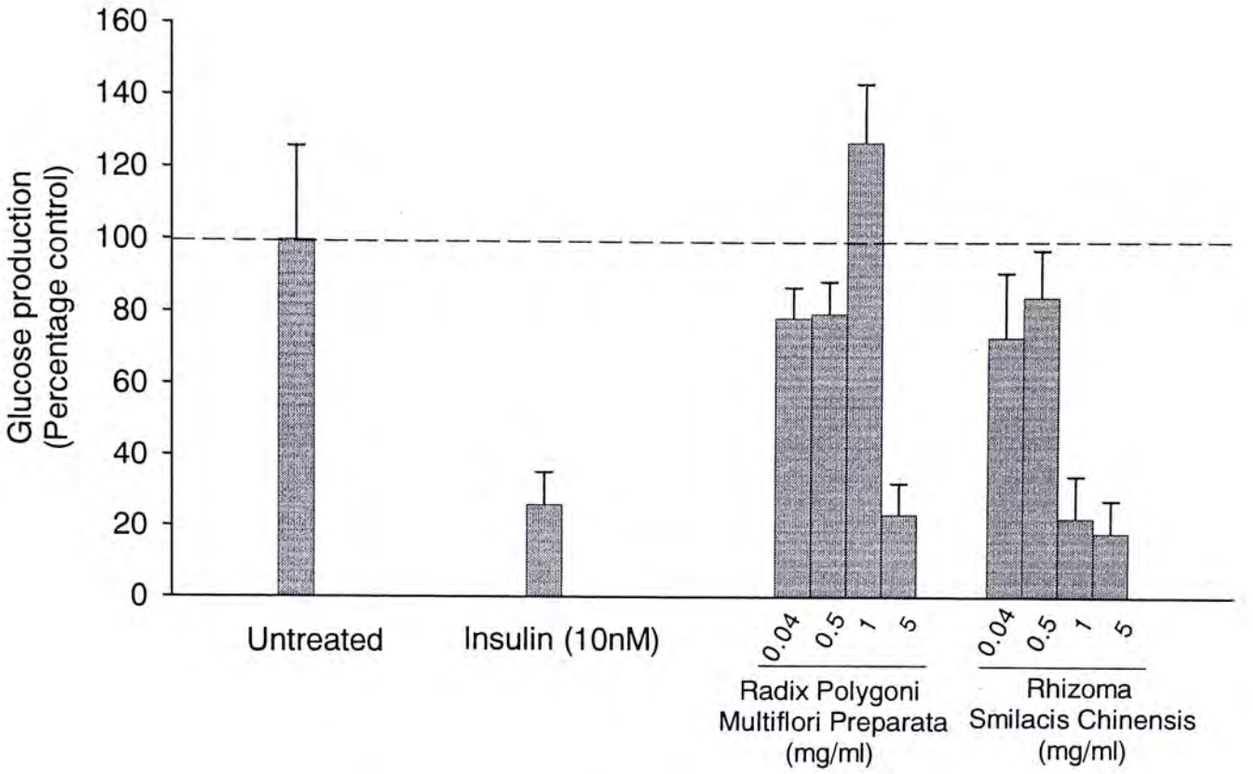


Figure 3.7. Effects of Rhizoma Smilacis Chinensis and Radix Polygoni Multiflori Preparata treatment on glucose production in H4IIE hepatoma cells. Cells were incubated with DEX/pCPT-cAMP, without additional treatment, or with herbal extracts, or with 10nM of insulin, then glucose production buffer containing gluconeogenic precursors was added, followed by determination of glucose released into the glucose production buffer by enzymatic-spectrophotometric glucose oxidase/peroxidase assay. The amount of glucose produced was normalized by cell protein. The glucose production values of the herbal extract treated group were expressed as the percentage of the untreated control group. Data are expressed as mean + SEM (n = 3).

Herbal extracts	Percentage stimulation or inhibition of glucose production (compared to negative control)			
	Herbal extract concentration (mg/ml)			
	0.04	0.2	1	5
Formula 1	+2.5%	+4.3%	+5.5%	+18.7%
Radix Astragali (黃耆)	-9.5%	+76.5%	+115.6%	+23.8%
Radix Rehmanniae (生地)	+46.4%	+172.2%	+32.9%	+45.5%
Rhizoma Atractylodis Macrocephalae (白朮)	-8.5%	-26.2%	-17.1%	-10.0%
Radix Stephaniae Tetrandra (漢防己)	-1.7%	+5.7%	+1.3%	---

Table 3.1. Summary of the effects of the formula 1 and its component herbs (except Rhizoma Smilacis Chinensis and Radix Polygoni Multiflori Preparata) on glucose production in H4IIE hepatoma cells. The glucose production values of the herbal extract treated group were expressed as the percentage of the untreated control group. The percentage values represent the extent of stimulation or inhibition of glucose production at each concentration (n = 3). The glucose production assay of Radix Stephaniae Tetrandra at 5mg/ml was not performed because cytotoxicity was observed at this dosage.

To further confirm the effect of the herbs on gluconeogenesis and elucidate the possible mechanism of the herbal action on glucose production, PEPCK activity assay was performed. This time, with larger sample size, statistical analysis was performed. The result of the PEPCK assay is shown in Figure 3.8. Insulin treatment (10nM) showed 58% inhibition of PEPCK activity ($p<0.05$). Similar to the results of the glucose production assay, Rhizoma Smilacis Chinensis and Radix Polygoni Multiflori Preparata significantly inhibited the PEPCK activity only at 5mg/ml by 52.0% and 42.6% respectively ($p<0.05$). Interestingly, Rhizoma Smilacis Chinensis did not show significant decrease in PEPCK activity at 1mg/ml; however, about 80% of inhibition on glucose production was observed at this dosage. Consistent with the glucose production assay result, Radix Polygoni Multiflori Preparata showed mild activation (33.0%, $p<0.05$) on the PEPCK activity at 1mg/ml and both herbs showed no effect on PEPCK activity at lower dosages.

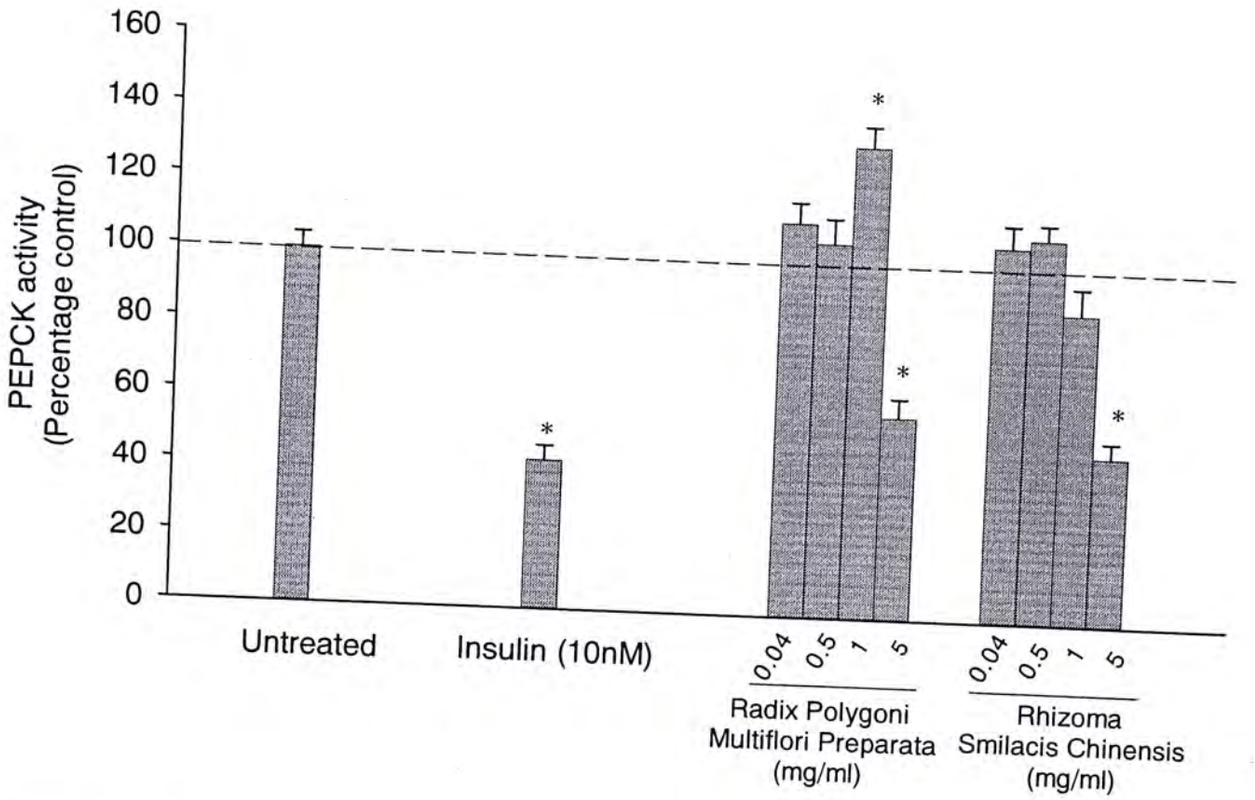


Figure 3.8. Effects of Rhizoma Smilacis Chinensis and Radix Polygoni Multiflori Preparata treatment on PEPCK activity in H4IIE hepatoma cells. Cells were incubated with DEX/pCPT-cAMP, without additional treatment, or with herbal extracts, or with 10nM of insulin. Cells were then lysed and the lysate was mixed with PEPCK assay buffer containing NADH to measure the PEPCK activity by the rate of NADH consumption using spectrophotometric method. The PEPCK activity was normalized by cell protein. The PEPCK activities of the herbal extract treated group were expressed as the percentage of the untreated control group. Data are expressed as mean + SEM (n = 8 - 9). * Significant difference between untreated control and herbal extract or insulin treated groups ($p < 0.05$).

3.5.3. Glucose uptake assay in BBMV

Chloroform extraction on the herbal water extracts was performed prior to the BBMV glucose uptake assay. From the result shown in Table 3.2, the yield of extraction was low (0.2% to 0.7%) in most of the herbs, except Radix Stephaniae Tetrandra (漢防己), which had the percentage yield of 3.2%. The chloroform extraction was carried out to remove the glucose content in the water extract because the trace amount of glucose in the herbal water extract would affect the uptake reaction by the BBMV. However, chloroform extraction would also lead to the loss of the polar compounds from the herbal extracts.

Since a number of batches of BBMV were prepared, to confirm the functionality of each batch of BBMV, a quality control experiment on the glucose uptake time profile was performed. Show in Figure 3.9, a typical bell-shaped curve on the glucose uptake profile was obtained, showing that the glucose uptake of BBMV achieved maximum at 20 seconds and after that, the glucose trapped in the BBMV gradually decreased (Kessler *et al.*, 1978; Semenza *et al.*, 1984). The transient overshoot of the glucose influx at the initial reaction was due to the sodium ion gradient across the brush border membrane. As the sodium gradient faded out, the glucose concentration inside and outside the BBMV gradually attained equilibrium (3600 seconds). Only the batches of BBMV with the bell-shaped glucose uptake profile similar to Figure 3.9 were used for the subsequent experiments.

Herbs	Yield	Equivalent aqueous extract concentration (mg/ml) to 1mg/ml of chloroform extract
Formula 1	0.5%	200
Radix Astragali (黃耆)	0.2%	500
Radix Rehmanniae (生地)	0.2%	500
Rhizoma Smilacis Chinensis (菝葜)	0.3%	333.33
Rhizoma Atractylodis Macrocephalae (白朮)	0.7%	142.86
Radix Polygoni Multiflori Preparata (制首烏)	0.3%	333.33
Radix Stephaniae Tetrandra (漢防己)	3.2%	31.25

Table 3.2. The percentage yield of the chloroform extraction from the herbal aqueous extract and the equivalent aqueous extract concentration to 1mg/ml of chloroform extract. The extraction yield was calculated by the ratio of the weight of chloroform extraction product to the weight of water extract used for chloroform extraction.

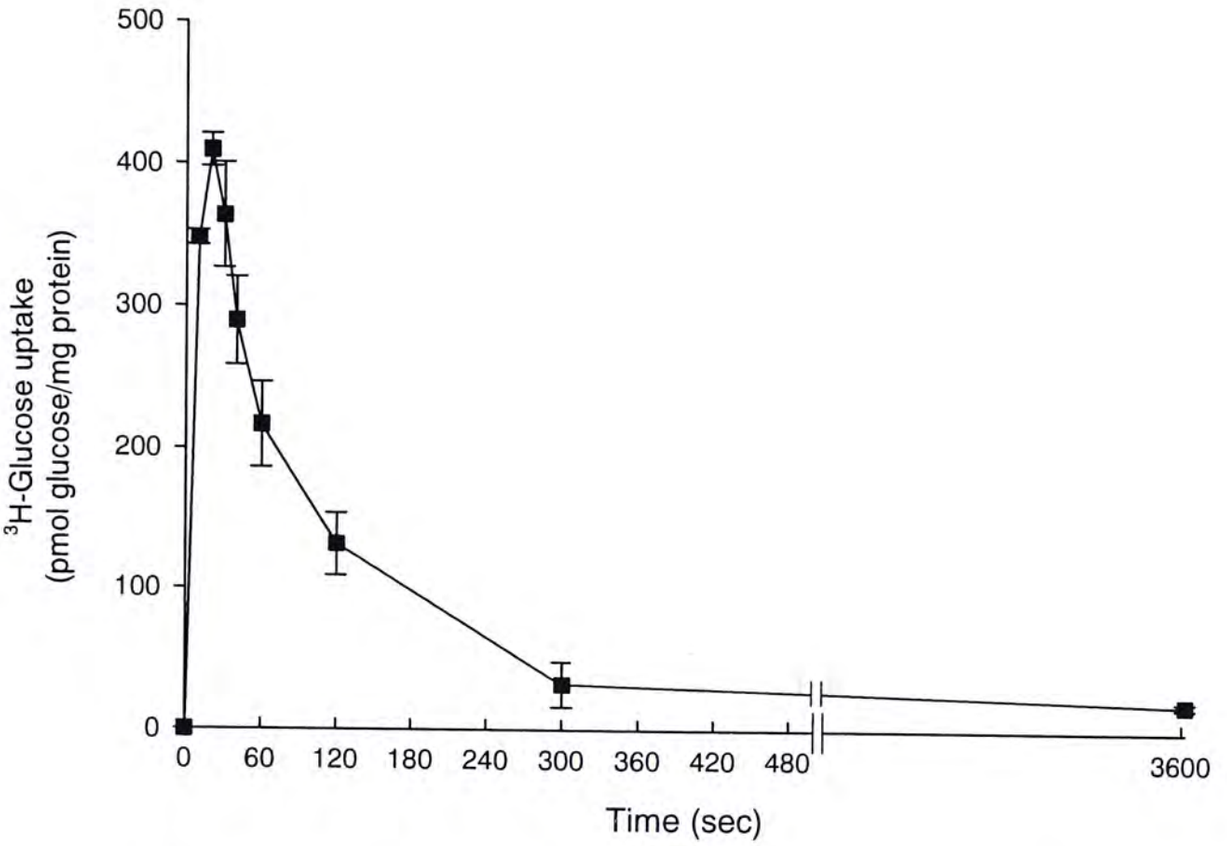


Figure 3.9. The glucose uptake profile of BBMV. BBMV were mixed with ^{3}H-glucose at room temperature and the glucose uptake reaction was stopped at different times by the addition of stop-wash buffer. The amount of radioactive glucose influx was normalized by the vesicles protein concentration. Data are expressed as mean ± SEM (n = 11 - 12).

To effectively inhibit glucose absorption from the intestine, the substance should be able to inhibit the absorption during the maximal glucose uptake, i.e. at 20 seconds. Glucose transport across the brush border membrane is primarily unidirectional up to this time point. Moreover, detecting the inhibition at the maximum point can increase the sensitivity of the detection of the inhibitory effect. Therefore, in the subsequent experiments, the drugs were screened for their inhibitory effects on BBMV glucose uptake at 20 seconds of reaction.

From the herbal extract experiments (Figure 3.10), four out of the seven herbs tested showed significant inhibitory effect on glucose absorption at the dosage of 1mg/ml chloroform extract. The four effective extracts included Formula 1, Radix Astragali (黃耆), Rhizoma Atractylodis Macrocephalae (白朮) and Radix Stephaniae Tetrandra and they showed 42.3%, 35.4%, 66.3% and 44.1% of inhibition, respectively ($p<0.05$).

Interestingly, when the dosage effects of formula 1, Radix Astragali and Rhizoma Atractylodis Macrocephalae were tested, all the herbal chloroform extracts showed dose-dependent inhibition on the glucose uptake (Figure 3.11).

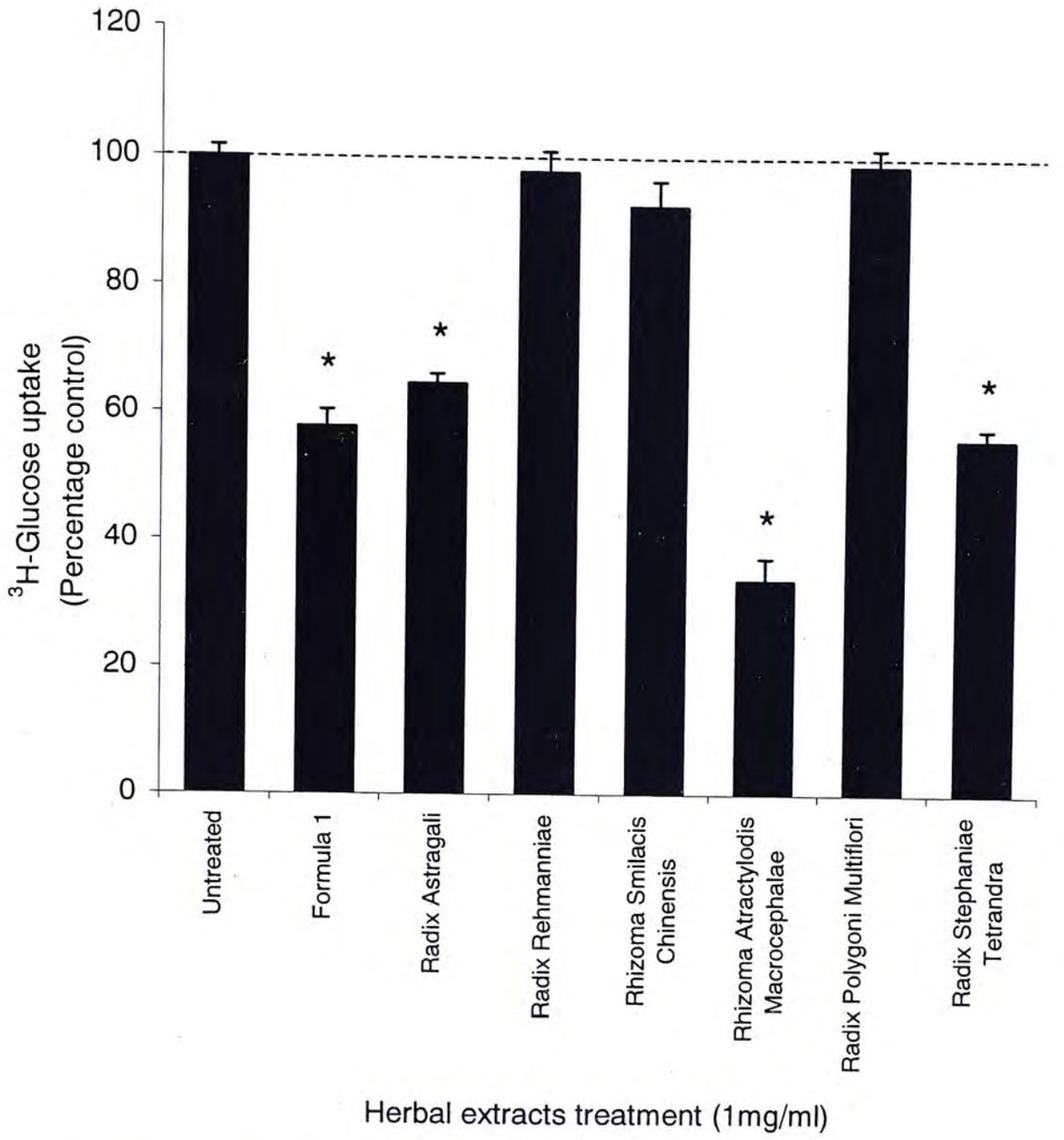


Figure 3.10. Effects of the chloroform extracts of the herbal water extracts on glucose uptake of BBMV. BBMV were mixed with ^{3}H-glucose, without additional treatment, or with herbal chloroform extracts (1mg/ml), at room temperature and the glucose uptake reaction was stopped at 20sec by the addition of stop-wash buffer. The amount of radioactive glucose influx was normalized by the vesicle protein concentration. Data are expressed as mean + SEM (n = 11 - 12). * Significant difference between untreated control and herbal extract treated groups ($p < 0.05$).

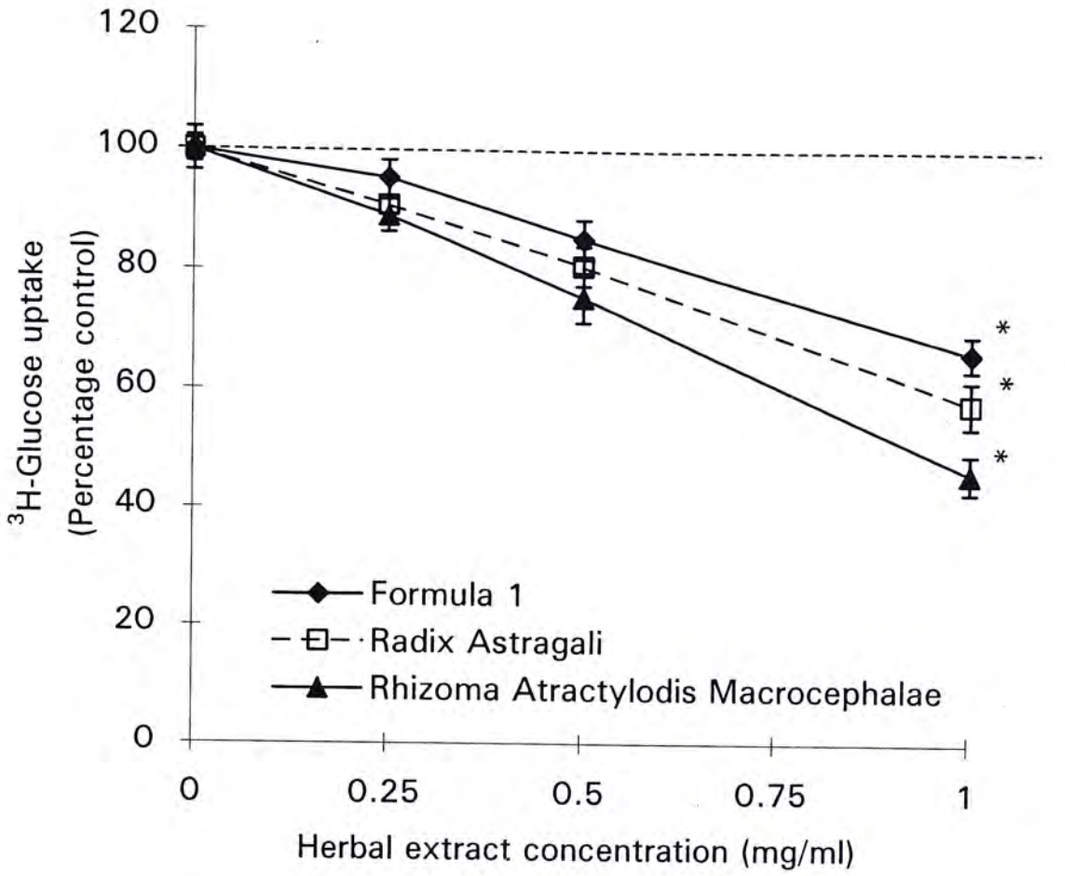


Figure 3.11. The dose-dependent inhibition of BBMV glucose uptake by formula 1, Radix Astragali and Rhizoma Atractylodis Macrocephalae. BBMV were mixed with ^{3}H-glucose, without additional treatment, or with herbal chloroform extracts, at room temperature and the glucose uptake reaction was stopped at 20sec by the addition of stop-wash buffer. The amount of radioactive glucose influx was normalized by the vesicles protein concentration. Data are expressed as mean $\pm$ SEM ($n = 8 - 9$). * Significant difference between untreated control and herbal extract treated groups ($p < 0.05$).

3.6. Discussion

3.6.1. Glucose uptake in 3T3-L1 adipocytes and Hs68 fibroblasts

All herbal water extracts except Radix Stephaniae Tetrandra (漢防己) demonstrated stimulatory effects on glucose uptake in 3T3-L1 adipocytes and Hs68 skin fibroblasts. These observations imply the possibilities that the effective herbal extracts may also promote glucose uptake *in vivo* and improve glucose clearance from the bloodstream. The stimulatory effect of Radix Rehmanniae (生地) in Hs68 fibroblasts was in a dose-dependent manner. Other herbs such as Rhizoma Smilacis Chinensis (菝葜) and Radix Polygoni Multiflori Preparata (制首烏) stimulated glucose uptake in lower dosages but inhibited glucose uptake at high dosage (10mg/ml). These observations indicate that these herbal extracts are effective at different dose ranges. In addition, the effects of the herbal extracts were different in the two cell lines. For example, Radix Astragali (黃耆) stimulates glucose uptake in Hs68 fibroblasts at 10mg/ml but inhibits the uptake in 3T3-L1 adipocytes at this concentration. This implies that the effects of the herbal extracts were also tissue-specific.

A wide range of compounds were shown to have stimulatory effect on 3T3-L1 adipocytes glucose uptake, for example, arachidonic acid (Nugent *et al.*, 2001) and endothelin (Wu-Wong *et al.*, 1999). Thiazolidinediones have been targeted to the adipose tissue to increase the glucose uptake and decrease the insulin resistance in diabetic patients (Arner, 2003). Traditional Chinese herbal medicines, such as Radix Ginseng and Radix Asparagi, also possess similar activities (Hong *et al.*, 2000). The herbal extracts which demonstrated stimulatory effect on the glucose uptake in the 3T3-L1 adipocytes in this study

could potentially improve glucose transport in the adipose tissue *in vivo*. However, further experiments are needed to such this effect.

Although skin fibroblast is not a major insulin responsive tissue, it is believed that the stimulation in the basal glucose uptake in insulin non-responsive tissues can also enhance the glucose clearance from the bloodstream (Gherzi *et al.*, 1992). Moreover, the glucose uptake into fibroblasts may give insight to the therapeutic effects of these herbal extracts on diabetic foot ulcer. One of my colleagues is working on the effects of these herbal extracts on cell proliferation. Preliminary results showed that Radix Astragali, Radix Rehmanniae (生地) and Rhizoma Atractylodis Macrocephalae (白朮) stimulated proliferation of skin fibroblast cell line. Moreover, the dosages which showed positive results in the glucose uptake experiment and the proliferation experiment are approximately the same. However, not all herbal extracts that stimulated glucose uptake could promote cell proliferation. This indicates that glucose uptake is not the sole factor affecting the cell proliferation (Lau *et al.*, unpublished data).

The effects of the herbs on glucose transport observed in the two cell types were probably mediated by glucose transporters. In the 3T3-L1 adipocytes, Glut1 and Glut4 are expressed. The herbal extracts may possess insulin-like effects on the 3T3-L1 adipocytes and stimulate the translocation of the Glut4 proteins from the intracellular vesicles to the plasma membrane (Harrison *et al.*, 1992). Translocation of Glut1 may also occur, but the extent of translocation is much less than that of Glut4 (Calderhead *et al.*, 1990). The stimulation in

glucose uptake observed in Hs68 fibroblast was most likely mediated by the activation of Glut1 and Glut3 proteins on the plasma membrane (Longo *et al.*, 1992). However, the stimulation observed in glucose uptake in both cell lines was likely not mediated by the translational activation of glucose transporters. It is because the cells were incubated with the herbal extracts for 30 minutes, but the translational activation of glucose transporters usually takes more than 2 hours (McGowan *et al.*, 1995). Nevertheless, further experiments are required to confirm the involvement of different glucose transporters in mediating the effects of different herbs.

Radix Stephaniae Tetrandra was cytotoxic to the 3T3-L1 adipocytes and Hs68 fibroblasts at 1mg/ml and 10mg/ml (Data not shown). No toxicity of this herb was previously reported. Moreover, a number of herbal extracts tested showed inhibitory effect on the glucose uptake at higher concentrations. The inhibition of glucose uptake may be caused by the disruption of pH of the extracellular environment during the herbal extract incubation. At the dosage that glucose uptake was inhibited, there was no obvious cell death observed. Further experiments are needed to confirm the cytotoxicity of these herbs. Apart from the cytotoxicity, the glucose present in the herbal extracts may also be responsible for the inhibition of the glucose uptake at higher herbal extract concentrations. However, since the cells were washed extensively after herbal extract incubation and before radioactive glucose incubation, the glucose present in the herbal extracts should not directly compete with the radioactive glucose for the glucose transporters. Furthermore, such inhibition was not exclusively observed in herbal extracts with high glucose content. For example, the glucose content of Radix Astragali extract was lower than Radix Rehmanniae, but

glucose uptake inhibition was only observed in Radix Astragali at 10mg/ml but not in Radix Rehmanniae in 3T3-L1 adipocytes. Therefore, the glucose content in the herbal extracts cannot fully explain the inhibition on glucose uptake observed at high herbal extract concentration.

In the future, mechanistic study of the stimulation of glucose uptake in the two cell lines can be conducted. Stimulation of glucose uptake in adipocytes may be mediated by the translocation of Glut4 from intracellular vesicles to plasma membrane. To confirm this effect of the active herbal extracts, western blot analysis on the amount of Glut4 on the plasma membrane using subcellular fractionation can be conducted (Perrini *et al.*, 2004). Alternatively, Glut4-GFP (green fluorescence protein) fusion construct can be employed to study the subcellular localization of Glut4 after herbal extract treatment (Powell *et al.*, 1999). Moreover, the herb-insulin interaction can be studied by the insulin-stimulated glucose uptake in the 3T3-L1 adipocytes to elucidate the interaction between insulin and the herbal extracts (Kamei *et al.*, 2002). For the stimulation of glucose uptake in Hs68 fibroblasts, since the incubation time of herbal extracts with cells was only 30 minutes, it is not likely that the extracts would increase the glucose uptake by activating translation level of Glut1. To confirm the effect of the herbal extracts on Glut1, protein synthesis inhibitors can be used (Khayat *et al.*, 1998).

3.6.2. Glucose production and PEPCK activity in H4IIE hepatoma cells

Rhizoma Smilacis Chinensis (菝葜) and Radix Polygoni Multiflori Preparata (制首烏) inhibited the glucose production, accompanied by the inhibition of the PEPCK activity in H4IIE cells.

In the functional assay of PEPCK, both herbs showed about 50% of inhibition on the PEPCK activity at 5mg/ml, which is less than the extent of inhibition in glucose production assay. Moreover, the PEPCK activity was not inhibited by 1mg/ml of Rhizoma Smilacis Chinensis (菝葜) while about 80% of inhibition on glucose production was observed at this dosage. The results indicated that these two herbs may also inhibit gluconeogenesis through other mechanisms, e.g. glucose-6-phosphatase (G6Pase) activity, since PEPCK and G6Pase are the two major controlling enzymes in the gluconeogenesis pathway (Lochhead *et al.*, 2000). G6Pase is one main target in current diabetic medication (Davies *et al.*, 1999). Therefore, the effects of the herbs on G6Pase activity deserve further investigation.

The inhibitory effect of Rhizoma Smilacis Chinensis and Radix Polygoni Multiflori Preparata on glucose production was observed at 5mg/ml. It is difficult to justify that this high concentration of the herbal extract would be present in the human liver. It can only be speculated that the herbal extract concentration in the liver is higher than elsewhere in the body because the digestion products from the extracts firstly reach the liver after absorption from the intestine. For example, research found that the exposure of the liver to

metformin is about five times more than that in the systemic circulation (Stepensky *et al.*, 2002). Inhibition of glucose production and PEPCK activity observed in high herbal extract concentration would raise another concern that the inhibition observed was the result of cytotoxicity, since it was found that cytotoxic chemicals would also decrease gluconeogenesis activity (Ashida *et al.*, 2000). However, no sign of cell death was observed after five hours of herbal extract incubation.

Apart from metformin and some other pure compounds, a few herbs and their pure components have been shown to have inhibitory effect on the H4IIE glucose production. For example, epigallocatechin gallate, a constituent of green tea, has been shown to mimic the insulin effect by activating mitogen-activated protein kinase cascade and the phosphatidylinositol 3-kinase pathway. Both pathways lead to the suppression of PEPCK and G6Pase gene transcription (Barthel and Schmoll, 2003).

The H4IIE cell line provides a simple model for the test on glucose production. However, in the present study, the interaction between herbs and hormones were not studied. Since many hormones are involved in maintaining hepatic glucose production in the body, the herb-hormone interaction may be studied in the future by co-incubating the hepatoma cells with hormones and herbal extracts.

In summary, Rhizoma Smilacis Chinensis and Radix Polygoni Multiflori Preparata extracts inhibited glucose production as well as PEPCK activity in

H4IIE hepatoma cells. Such observations imply the possibilities that these herbal extracts may also inhibit hepatic glucose production *in vivo*.

3.6.3. Glucose absorption in BBMV

Formula 1, Radix Astragali (黃 耆), Rhizoma Atractylodis Macrocephalae (白朮) and Radix Stephaniae Tetrandra (漢防己) inhibited Sglt1 activity in the BBMV and except Radix Stephaniae Tetrandra, the inhibitory effects on the glucose uptake were dose-dependent.

When the dosage of the chloroform extract is converted back to the equivalent aqueous extract by the percentage yield of chloroform extraction, the dosage is much higher than that used in other *in vitro* assays (Table 3.2). Alternatively, a bioequivalence study can be performed between crude herbal extracts and chloroform extracts so that the effective dosage of the herb could be determined. Radix Stephaniae Tetrandra (漢防己) is the most potent among the others since its effective dose is much lower than the other effective herbs.

Chloroform extraction on the herbal water extracts were performed because it was found that the herbal extracts contained glucose from the HPLC analysis in section 2.5.3. The glucose content in the herbal water extracts would interfere with the glucose uptake of the BBMV and give false positive results. In the BBMV glucose uptake assay, BBMV was incubated with herbal extract together with radioactive glucose solution and the final glucose concentration in the reaction mixture was 0.067mM, or equivalent to 0.012mg/ml. The glucose content in the herbal water extract was comparable to that in the BBMV assay

solution. For example, Rhizoma Atractylodis Macrocephalae, which contained the least amount of glucose among all (0.66% w/w), the concentration of glucose in 1mg/ml herbal water extract solution would be 0.0066mg/ml, which is about half of the value of the glucose concentration in the BBMV assay. Therefore, it was necessary to remove the glucose from the water extracts. However, chloroform extraction inevitably causes the loss of polar substances which may also possess such inhibitory effect on the transporter. At present, there is no effective method to eradicate glucose without affecting the content of other chemicals from the extracts.

The yield of chloroform extraction was generally very low. Except Radix Stephaniae Tetrandra, the percentage yields of the other herbal extract were less than 1%. It was because the starting materials of chloroform extraction were the water extract of the herbal materials. It indicates that most of the non-polar substances in the raw herbal materials were removed by the water extraction and most of the compounds in the water extracts were polar.

The inhibition of these herbal extracts was possibly caused by direct interaction with the Sglt1 protein (Klaren *et al.*, 2000). Apart from the direct interaction, the change of the membrane fluidity of the vesicles was another possible mechanism for the inhibition (Proulx, 1991). For future experiments, these herbal extracts can be tested for their effects on the Mg^{2+}-ATPase which would affect the membrane fluidity (Kitagawa *et al.*, 1995). BBMV provides a simple model for rapid screening of the herbal extracts on the Sglt1. The vesicle contains no enzymes and ribosomes; therefore, it has no transcriptional and translational modification of the transporter. Any effect observed is likely to be

the direct interaction of the substance to the transporter. However, this model does not allow for the examination of the regulatory mechanisms on the transporters and other factors affecting the intestinal glucose uptake, e.g. the effect on α-glucosidase.

In summary, the chloroform extracts of formula 1, Radix Astragali, Rhizoma Atractylodis Macrocephalae and Radix Stephaniae Tetrandra water extracts inhibited Sglt1 activity in the apical membrane of rabbit small intestinal enterocytes shown by the BBMV model. These observations imply the possibilities that these herbal extracts may inhibit the intestinal glucose absorption and ameliorate postprandial hyperglycaemia *in vivo*.

3.6.4. Conclusion

The *in vitro* studies attempted to simulate the individual tissues in the body which are targeted by current anti-diabetic medication. The glucose homeostasis regulation of these tissues by the herbal extracts may give implications on the regulation of the systemic glucose level in the body (Figure 3.1). Table 3.3 summarizes the effects of the herbs in the *in vitro* models. In the Hs68 fibroblast and the 3T3-L1 adipocyte systems, increase in the glucose uptake was desirable while in the H4IIE hepatoma cells and the BBMV systems, decrease in the glucose production and absorption were the desirable effects, respectively. Increase in glucose uptake in 3T3-L1 adipocytes and Hs68 fibroblasts indicate that the herbal extracts may enhance glucose clearance from the bloodstream (Szalkowski *et al.*, 1995). Inhibition of the glucose production in H4IIE hepatoma cells indicates that the herbal extracts may reduce the glucose input into the bloodstream (Lochhead *et al.*, 2001). Inhibition of BBMV glucose uptake implies the effects of the herbal extracts on inhibiting intestinal glucose absorption and ameliorating postprandial hyperglycaemia (Creutzfeldt and Folsch, 1983).

Formula 1, Radix Astragali (黃耆), Rhizoma Smilacis Chinensis (菝葜), Rhizoma Atractylodis Macrocephalae (白朮) and Radix Polygoni Multiflori Preparata (制首烏) showed positive results in at least three of our four *in vitro* screening systems. Rhizoma Smilacis Chinensis was the most powerful herb according to our *in vitro* screening systems. This herb was highly active in promoting glucose uptake in Hs68 fibroblasts and 3T3-L1 adipocytes and inhibiting glucose production in H4IIE hepatoma cells. Therefore, Rhizoma

Smilacis Chinensis was chosen for further investigation on its anti-diabetic effects *in vivo*. The effect of formula 1 was also studied in the *in vivo* system due to its relevance to the clinical study. In addition, formula 1 was also highly active in inhibiting glucose absorption in BBMV and stimulating glucose uptake in 3T3-L1 adipocytes and Hs68 fibroblasts.

	Hs68 Glucose uptake	3T3-L1 Glucose uptake	H4IIE		BBMV Glucose uptake
			Glucose production	PEPCK activity	
Formula 1	+ 58.6% (1.0) *	+ 63.6% (0.1) *	+ 2.5% (0.04)	---	- 42.3% (1.0) *
Radix Astragali (黃耆)	+ 40.1% (1.0) *	+ 94.6% (0.1) *	- 9.5% (0.04)	---	- 35.4% (1.0) *
Radix Rehmanniae (生地)	+ 33.0% (10.0) *	+ 78.3% (0.01) *	+ 32.9% (1.0)	---	-2.05% (1.0)
Rhizoma Smilacis Chinensis (菝葜)	+ 51.8% (0.01) *	+ 87.7% (0.1) *	- 81.9% (5.0) *	- 52.0% (5) *	-7.36% (1.0)
Rhizoma Atractylodis Macrocephalae (白朮)	+ 26.6% (0.01) *	+ 43.3% (0.1) *	- 26.2% (1.0)	---	- 66.3% (1.0) *
Radix Polygoni Multiflori Preparata (制首烏)	+ 43.3% (1.0) *	+ 55.4% (0.01) *	- 76.7% (5.0) *	- 42.6% (5) *	-1.12% (1.0)
Radix Stephaniae Tetrandrae (漢防己)	- 54.2% (0.001) *	+ 11.0% (0.1)	- 5.7% (0.2)	---	- 44.1% (1.0) *

Table 3.3. The summary of the effects of the formula 1 and its component herbs in the *in vitro* screening systems. The percentage values represent the greatest extent of stimulation or inhibition in each assay at the concentration indicated in the brackets in mg/ml water extract for all assays except for BBMV studies in which chloroform extracts of water extracts were used. * Significant difference between untreated control and herbal extract treated groups ($p < 0.05$).

Chapter 4: The anti-diabetic effects of formula 1 and Rhizoma Smilacis Chinensis *in vivo*

4.1 Introduction

Rhizoma Smilacis Chinensis, the herb which gave the most promising results in the *in vitro* screening systems, as well as formula 1, were further tested for their anti-diabetic effects in an animal model.

4.1.1 Diabetic animal models

Diabetes mellitus is a multifactorial disorder characterized by hyperglycaemia. This project focuses on type 2 diabetes. Currently, there are a number of animal models of type 2 diabetes available; however, it must be noted that these models differ significantly from each other and none of them is equivalent to the human diabetes (Bell and Hye, 1983). Therefore, the characteristics and the limits of each model should be recognized and the animal model should be chosen according to the research interests.

Rats and mice are the most commonly used animal models for the diabetes study because they are easier to manipulate as compared to larger animals. Therefore, only the models using rats and mice are described. At present, two major types of diabetic rat and mice models are being used for investigation (Mcintosh and Pederson, 1999):

(a) Genetically manipulated animals;

(b) Chemically manipulated animals.

(a) Genetically manipulated rat and mice models

From a number of epidemiological and twin studies, genetic predisposition of the individual is an important aetiological factor for type 2 diabetes in human (Froguel and Hager, 1995). Studies have revealed mutations in a number of candidate genes including those for insulin, insulin receptor, glucokinase and mitochondrial tRNA (Elbein, 1997). The genetic rodent models of diabetes provide important insights into the aetiology of this disorder. Some genetic rodent models are monogenic, such as *db*/*db* mice (Dong *et al.*, 1997) and Zucker diabetic fatty rat (Zawalich *et al.,* 1995). Some of them are polygenic, such as the Japanese KK mice (Mcintosh and Pederson, 1999), and the others are transgenic or knockout mice, such as IR/IRS-$1^{+/-}$ mice (Mauvais-Jarvis *et al.*, 2002). However, none of these models can adequately address the aetiologic and genetic conditions of diabetes. For example, *ob*/*ob* and *db*/*db* mice differ in many aspects from the typical diabetic patients and few patients exhibit the degree of obesity that characterizes these mice (Luo *et al.*, 1998).

(b) Chemically manipulated rat and mice models

Common chemically manipulated rodent diabetic models make use of cytotoxic chemicals specific to pancreatic β-cells, such as alloxan and streptozotocin (STZ), to induce diabetes. The damages in the endocrine pancreas would create a deficiency or a complete loss of insulin secretion, depending on the time of chemical induction (Rodrigues *et al.*, 1999). Diabetes can be induced by neonatal injection of alloxan or streptozotocin into rats (Kodama *et al.*, 1993), developing an insulin-deficient state and creating a mild and stable hyperglycaemic condition in the rats (Portha *et al.*, 1979). Insulin resistance is

also found in these chemically induced diabetic animals. The co-existence of insulin resistance and impaired insulin action endows this neonatally chemically induced rats a model of diabetes (Blondel *et al.*, 1989).

4.1.2 Neonatal streptozotocin-induced diabetic rat model

In this project, neonatal streptozotocin-induced diabetic rat model was used as the animal model for the study of the anti-diabetic effects of the herbal extracts. This model was first described by Portha *et al* (1974) and the diabetic condition was created by a single dose of streptozotocin via intravenous (i.v.) injection. Since then, the induction method has been modified and now, the regime of a single dose of intraperitoneal (i.p.) injection of STZ into neonatal rats is commonly used (Rodrigues *et al.*, 1999).

STZ is a cytotoxic chemical specific to pancreatic β-cells isolated from *Streptomyces achromogenes*. Structurally, streptozotocin contains a glucose-like moiety (Figure 4.1) that enables it to be selectively taken up by the pancreatic β–cells via glucose transporter type 2 (Glut2) (Schnedl *et al.*, 1994) and causing cell death after its entry. Although the exact mechanism of cell death is still unknown, it is believed that the nitrosurea group of STZ can cause alkylation of DNA (Delaney *et al.*, 1995) and act as a nitric oxide donor. The nitric oxide produced when streptozotocin is metabolized in the β-cells causes DNA damage directly or via reactive oxygen species (Kroncke *et al.*, 1995). The reactive oxygen species can also trigger a number of disastrous consequences and lead to cell death (Takasu *et al.*, 1991). For example, they would cause inhibition of the

electron transport chain (Turk *et al.*, 1993) as well as ATP and NAD^+ depletion in the β-cells (Nukatsuka *et al.*, 1990).

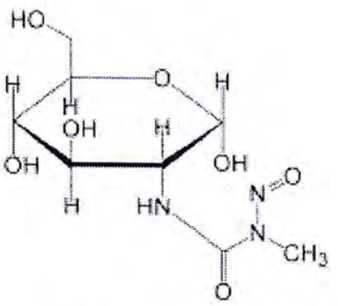


Figure 4.1. The chemical structure of streptozotocin. Streptozotocin contains a nitrosurea group linked to a glucose moiety that allows its entry into the pancreatic β-cells via Glut2.

Diabetes is created by the pancreatic β-cells damage caused by STZ in the neonates, followed by limited β-cells regeneration. At 6 to 22 weeks of age, persistent hyperglycaemia and impaired insulin secretion are found in the animals (Bonner-Weir *et al.*, 1981). Several regimes with different time and dosage of STZ injection have been developed. In this part of the project, two types of neonatal STZ-induced diabetic rat models were used, namely n0-STZ and n5-STZ. The n0-STZ model was created by introducing STZ (100mg/kg body weight) i.p. injection within 1 day after birth while the n5-STZ model was made by introducing the STZ (70mg/kg body weight) i.p. injection 5 days after birth.

Owing to the pancreatic β-cells destruction by STZ, these diabetic rats are marked by a decreased insulin stores and hence, hyperglycaemia and glucose intolerance (Weir *et al.*, 1981). However, the severity of the diabetic condition in these two models is quite different. Since the regeneration activity of the

pancreatic β-cells deteriorates with age, n0-STZ rats can regenerate more β-cells than n5-STZ rats and the diabetic condition of n5-STZ rats is more severe than that of the n0-STZ rats (McIntosh and Pederson, 1999). The pancreatic insulin content of the n5-STZ is 90% depleted at the age of 10 weeks while that of the n0-STZ is only 50% depleted at the same age (Blondel *et al.*, 1989). Therefore, the n0-STZ rats show only mild hyperglycaemia while the n5-STZ rats show more severe hyperglycaemia. However, insulin resistance is only found in n5-STZ rats but not in the n0-STZ rats (Blondel *et al.*, 1989; Kergoat and Portha, 1985).

The STZ-induced diabetic rat model was used in the study of the anti-diabetic effects of formula 1 and Rhizoma Smilacis Chinensis (菝葜). Formula 1 and Rhizoma Smilacis Chinensis were tested for their anti-diabetic effects in the n0-STZ and n5-STZ rat models by two tests, oral glucose tolerance test and basal glycaemia test. Oral glucose tolerance test monitors the anti-hyperglycaemic effect of the herbs after introducing herbal extract treatment followed by glucose challenge. Basal glycaemia test monitors the basal fasting plasma glucose following the herbal extract treatment to a fasting rat.

4.2 Objective

The objective of this part of the project is to evaluate the anti-hyperglycaemic and hypoglycaemic effects of formula 1 and Rhizoma Smilacis Chinensis on the oral glucose tolerance and basal glycaemic control in n0-STZ and n5-STZ diabetic rats, respectively.

4.3 Materials

4.3.1 Animals

Neonatal female albino Wistar rats were supplied by and kept at The Laboratory Animal Services Centre, The Chinese University of Hong Kong. Three to four rats were kept in a wire-bottomed cage and acclimated under the conditions of 22-25°C and a 12-hour light-dark cycle. The animals were supplied with standard rodent diet, Prolab 2500 rodent diet, and allowed free access to tap water. The animal study was approved by Animal Experimentation Ethics Committee under the project license number 02/022/MIS.

4.3.2 Chemicals and reagent kit

All chemicals were purchased from Sigma, St. Louis, MO, USA.

a) Streptozotocin (STZ)

Streptozotocin was dissolved in citrate buffer (0.028M of citric acid monohydrate and 0.255M of citric acid trisodium salt dehydrate, pH4.5) to the concentration of 20mg/ml. The STZ solution was freshly prepared before use.

b) Heparin sodium salt from porcine intestinal mucosa

Heparin was dissolved in distilled water to the concentration of 1250U/ml.

c) Glucose

Glucose was dissolved in distilled water to 400mg/ml for the oral glucose tolerance test. Brief sonication was performed to ensure complete dissolution.

d) Metformin

Metformin was dissolved in distilled water to the concentration of 200mg/ml for the oral glucose tolerance test and the basal glycaemia test.

e) Enzymatic-spectrophotometric glucose oxidase/peroxidase assay kit

Enzymatic-spectrophotometric glucose oxidase/peroxidase assay kit was purchased from Biosystems S.A., Spain. The assay kit includes a bottle of assay reagent, containing 70mmol/L of phosphate, 5mmol/L of phenol, more than 10U/ml of glucose oxidase, more than 1U/ml of peroxidase, 0.4mmol/L of 4-aminoantipyrine, at pH 7.5, and a bottle of glucose/urea/creatinine standard, containing 100mg/dl (5.55mmol/L) of glucose, 50mg/dl of urea, 2mg/dl of creatinine.

4.4 Methods

4.4.1 Induction of diabetes in rats

The n0-STZ diabetic rats were induced according to protocol previously described by Barbera *et al* (1997). Freshly-prepared STZ solution (20mg/ml) was injected into one-day old female Wistar rats intraperitoneally (100mg/kg body weight) for the use in oral glucose tolerance test. The n5-STZ diabetic rats were induced according to previously described protocol (Gokhale *et al.,* 1998). Freshly-prepared streptozotocin solution (20mg/ml) was injected into five-day old female Wistar rats intraperitoneally (70mg/kg body weight) for the use in basal glycaemia test. At week 4 or 5, the rats were separated from their mothers. The rats were used for experiment at the age of week 10-12.

4.4.2 Oral glucose tolerance test

The n0-STZ diabetic rats at the age of week 10 to 12 were used in the oral glucose tolerance test. The diabetic condition was confirmed by the determination of fasting hyperglycaemia at 7.0mM or over in the adult rats. The herbal extracts treatment was given to the rats once daily for eight consecutive days. Oral glucose tolerance test was performed and plasma glucose level of the rats was determined on day 1 for the acute effect of the herb, and also on day 8 for the chronic effect of the treatment.

Rats were firstly randomized into various groups ($n \geq 5$): negative control group (water, 5ml/kg body weight), positive control group (metformin, 5ml/kg, 200mg/kg body weight), and herbal extract treatment groups (822mg/kg formula 1 or 400mg/kg Rhizoma Smilacis Chinensis (菝葜) extract, 5ml/kg body weight).

The procedure of oral glucose tolerance test is described as follows: Prior to the experiment, the rats were fasted for 2 hours. Then, 300μl of blood samples from the rats were collected from tail vein in a 1.5ml microfuge tube containing heparin (1250U/ml, 5μl/tube). Within 30 minutes after blood sample collection, plasma sample was collected by centrifugation of the whole blood sample at 2000 x g for 5 minutes and the supernatant plasma was transferred to another tube and stored on ice. Fifteen minutes after the blood collection, herbal extract, water, or metformin was introduced orally to the rats by force-feeding. Blood was again collected and plasma was obtained by centrifugation 15 minutes after the force-feeding. Glucose solution (400mg/ml, 2g/kg and 5ml/kg body weight) was then force-fed to the rats 15 minutes after the blood collection. This time point was designated as "0 minute". Blood samples were collected at appropriate time points over a period of 90 minutes after the glucose loading. The plasma glucose levels were measured by the enzymatic-spectrophotometric glucose oxidase/peroxidase assay described in section 4.4.4. Rats were kept unfed throughout the whole experimental period. Rats were given the same treatment from day 2 to 7 once daily. The oral glucose tolerance test was performed with the same procedure on day 8.

4.4.3 Basal glycaemia test

The n5-STZ diabetic rats at the age of week 10 to 12 were used in the basal glycaemia test. The diabetic condition was confirmed by the determination of fasting hyperglycaemia at 7.0mM or over in the adult rats. The herbal extracts treatment was given to the rats once daily for eight consecutive days. Basal glycaemia test was performed and plasma glucose level of the rats was determined on day 1 for the acute effect of the herb, and also on day 8 for the chronic effect of the treatment.

Rats were firstly randomized into various groups ($n \geq 5$): negative control group (water, 5ml/kg body weight), positive control group (metformin, 5ml/kg, 200mg/kg body weight), and herbal extract treatment groups (411mg/kg formula 1 or 400mg/kg Rhizoma Smilacis Chinensis (菝葜) extract, 5ml/kg body weight).

The procedure of basal glycaemia test is described as follows: Prior to the experiment, the rats were fasted for 2 hours. Then, 300μl of blood samples from the rats were collected from tail vein as described in section 4.4.2. Herbal extract, water, or metformin was introduced orally to the rats by force-feeding. Blood samples were collected at appropriate time points over a period of 360 minutes after the treatment. Plasma was collected immediately by centrifugation of the blood samples. The rats were kept unfed throughout the experimental period. The plasma glucose levels were measured by the enzymatic-spectrophotometric glucose oxidase/peroxidase assay described in section 4.4.4. Rats were given the same treatment from day 2 to 7 once daily. The basal glycaemia test was performed with the same procedure on day 8.

4.4.4 Plasma glucose level determination

The plasma glucose levels were measured by an enzymatic-spectrophotometric glucose oxidase/peroxidase assay kit. The assay was performed as follows: 1ml of the assay reagent was aliquoted to 1.5ml microfuge tubes and pre-warmed in a 37°C water bath. 10μl of the plasma sample was added to the reagent and then the solution was mixed thoroughly. The mixture was incubated at 37°C for 5 minutes. In every set of experiment, a blank and a standard were also carried out by adding 10μl of distilled water and 10μl of standard solution respectively. The absorbance of the plasma sample and the standard was measured at 500nm against the blank using a spectrophotometer. The glucose concentration in the sample was calculated using the following general formula:

Glucose concentration of the plasma sample

= (Absorbance of plasma sample / Absorbance of standard) x 100mg/dl

4.4.5 Statistical analysis

Area under the curve (AUC) for glucose level across time was calculated for each rat on each day. AUC was used as a summary variable for the comparison of efficacy between each herb/formula/drug and water. Multilevel modeling was used to examine the effectiveness of each herb/formula/drug in reducing AUC (and hence glucose level). The rats' AUC across days were filled to multilevel models with adjustments for weight. The public domain software *MIXREG* was used for fitting multilevel models. All statistical tests were two-sided, with a significant level of $p<0.05$.

4.5 Results

4.5.1 Oral glucose tolerance test

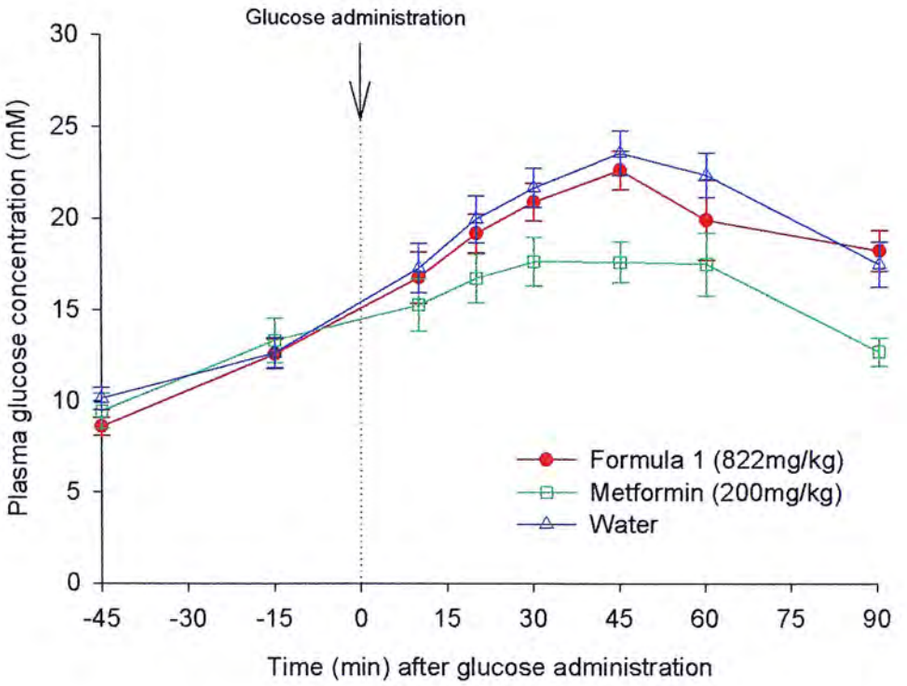
a) Formula 1

Formula 1 was tested for its anti-hyperglycaemic effect by oral glucose tolerance test in n0-STZ diabetic rats and the results are shown in Figure 4.2. The dosage of formula 1 used in the experiment was 822mg/kg and this was determined by twice of human equivalent dose. Metformin at the dosage of 200mg/kg was used as positive control. Statistical analysis was made by comparing area under the curve (AUC) of treatments with that of the water control.

On both days 1 and 8, no significant difference from water group was found in formula 1 treatment group. On the other hand, the positive control, metformin, significantly lowered the plasma glucose level on both days ($p<0.05$), thereby validating the model. Hence, it is concluded that formula 1 at the dosage of 822mg/kg did not have anti-hyperglycaemic effect on the n0-STZ rats.

After 8 days of treatment, body weights of rats in all groups increased gradually and no significant difference was found between treatment groups and water group (data not shown). No obvious side effect was observed with treatment groups.

A)



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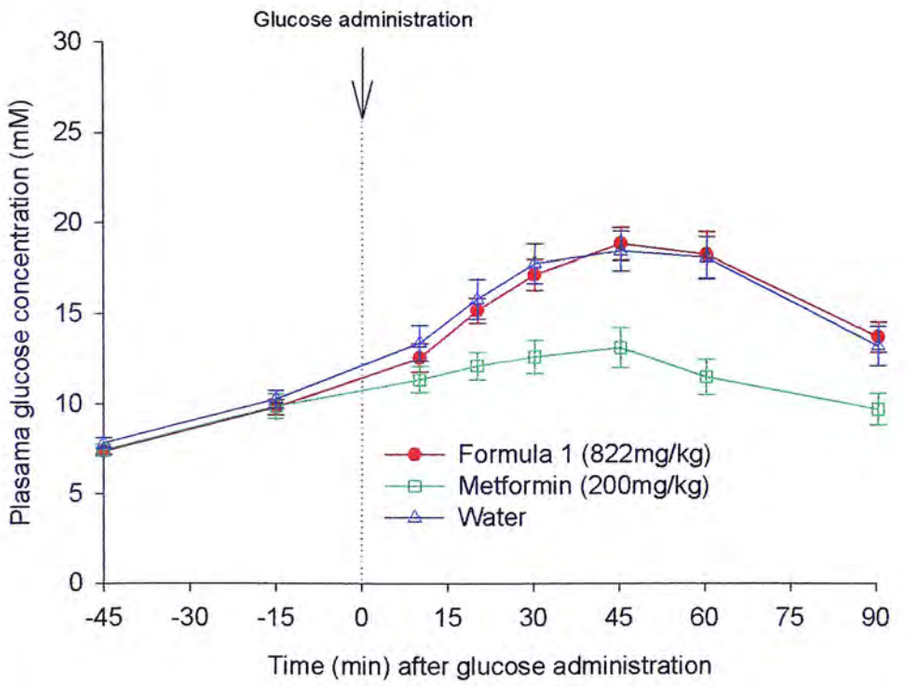


Figure 4.2. Oral glucose tolerance test of formula 1 (822mg/kg) treated animals on (A) day 1 and (B) day 8 experiment. Formula 1 (822mg/kg), or metformin (200mg/kg), or water, was administered orally to the n0-STZ diabetic rats after 2hr fasting and 30min before glucose load (2g/kg) (t = 0). Blood was taken at 10, 20, 30, 45, 60 and 90min after glucose administration. The treatment lasted for 8 consecutive days and oral glucose tolerance test was performed on day 1 and day 8 of the treatment. Data are expressed as mean ± SEM (n = 7 - 8).

b) Rhizoma Smilacis Chinensis (菝葜)

Rhizoma Smilacis Chinensis was tested for its anti-hyperglycaemic effect by oral glucose tolerance test in n0-STZ diabetic rats and the results are shown in Figure 4.3. The dosage of Rhizoma Smilacis Chinensis used in the experiment was 400mg/kg. Metformin at the dosage of 200mg/kg was used as positive control. Statistical analysis was made by comparing area under the curve (AUC) of treatments with that of the water control.

On both days 1 and 8, no significant difference from water group was found in Rhizoma Smilacis Chinensis treatment group. On the other hand, the positive control, metformin, significantly lowered the plasma glucose level on both days ($p<0.05$), thereby validating the model. Hence, it is concluded that Rhizoma Smilacis Chinensis at the dosage of 400mg/kg did not have anti-hyperglycaemic effect on the n0-STZ rats.

After 8 days of treatment, body weights of rats in all groups increased gradually and no significant difference was found between treatment groups and water group (data not shown). No obvious side effect was observed with treatment groups.

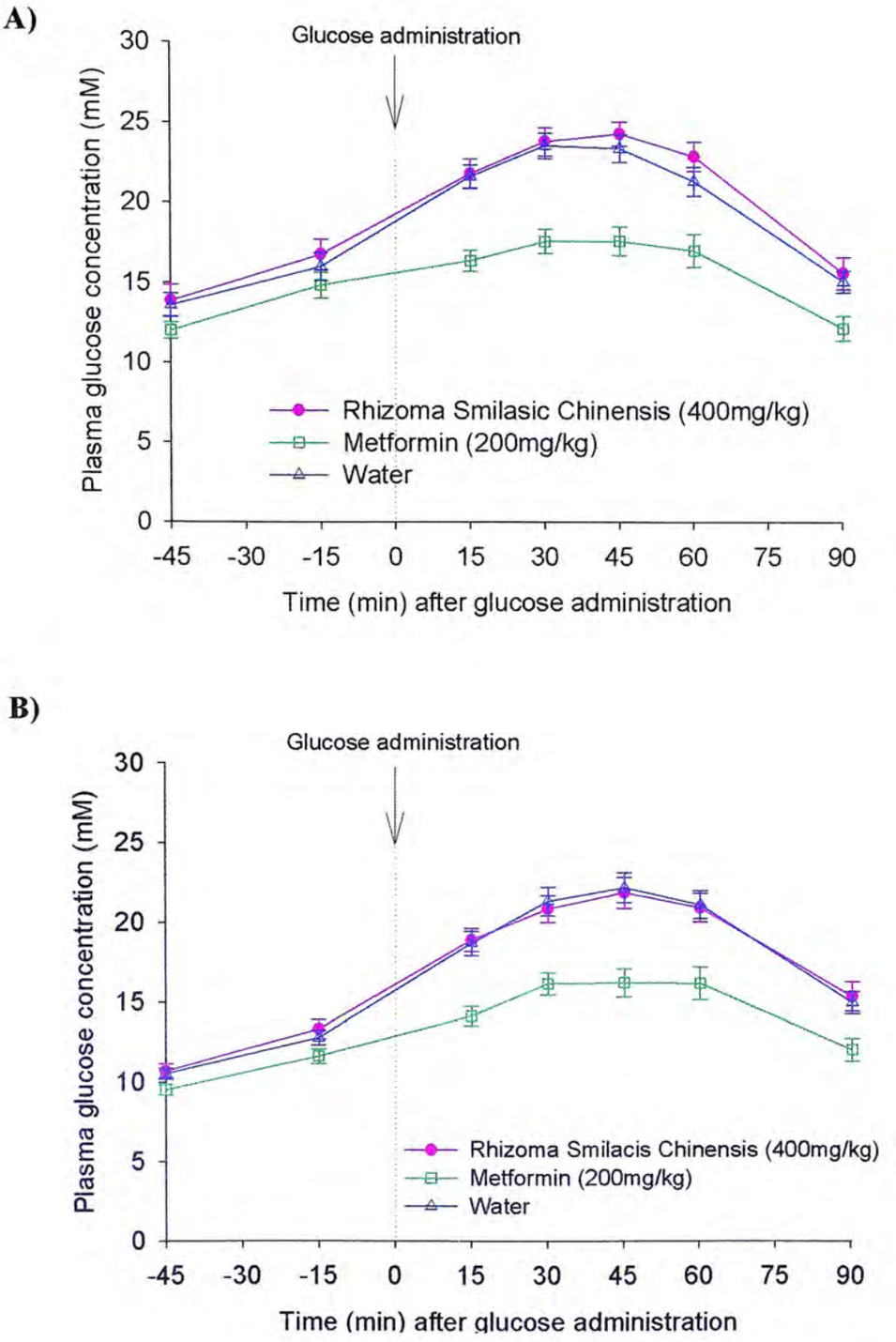


Figure 4.3. Oral glucose tolerance test of Rhizoma Smilacis Chinensis (400mg/kg) treated animals on (A) day 1 and (B) day 8 experiment. Rhizoma Smilacis Chinensis (400mg/kg), or metformin (200mg/kg), or water, was administered orally to the n0-STZ diabetic rats after 2hr fasting and 30min before glucose load (2g/kg) (t = 0). Blood was taken at 15, 30, 45, 60 and 90min after glucose administration. The treatment lasted for 8 consecutive days and oral glucose tolerance test was performed on day 1 and day 8 of the treatment. Data are expressed as mean $\pm$ SEM (n = 18 - 22).

4.5.2 Basal glycaemia test

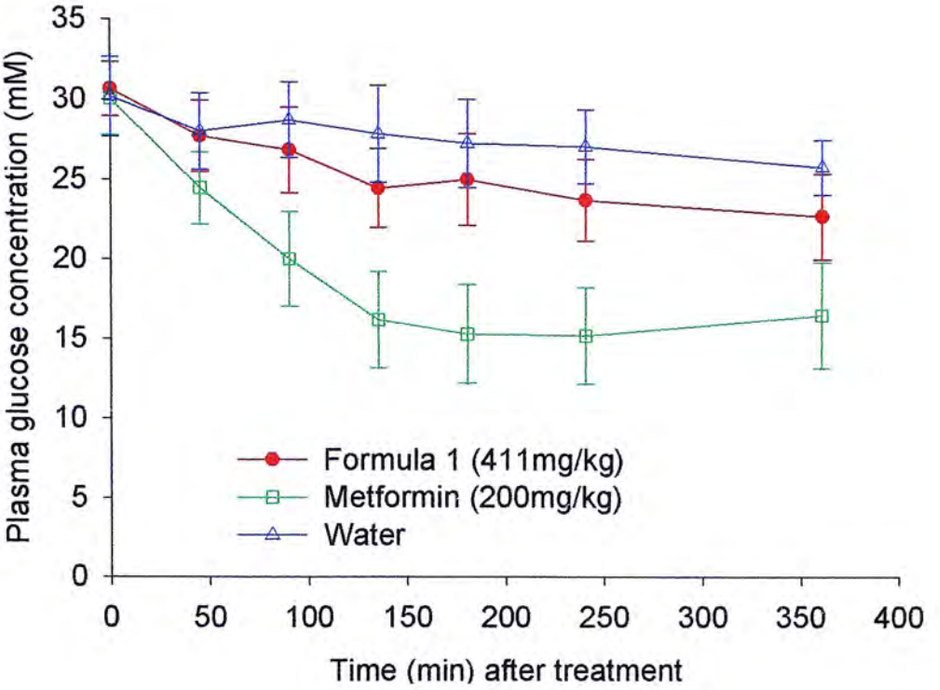
a) Formula 1

Formula 1 was tested for its effect on fasting glucose level by basal glycaemia test in n5-STZ diabetic rats and the results are shown in Figure 4.4. The dosage of formula 1 used in the experiment was 411mg/kg and it was determined by the human equivalent dose. Metformin (200mg/kg) was used as positive control. Statistical analysis was made by comparing area under the curve (AUC) of treatments with that of the water control.

Although no significant difference from water group was found in formula 1 treatment group on both days 1 and 8, the AUC value was about 8.6% and 8.1% lower than that of water control group on day 1 and day 8, respectively. The positive control, metformin, significantly lowered the plasma glucose level on both days ($p<0.05$), thereby validating the model. Hence, it is concluded that formula 1 at the dosage of 411mg/kg did not significantly lowered the fasting glucose on the n5-STZ rats. Experiment on n0-STZ rats with 822mg/kg formula 1 treatment was also performed and similar result was obtained (Appendix II).

After 8 days of treatment, body weights of rats in all groups increased gradually and no significant difference was found between treatment groups and water group (data not shown). No obvious side effect was observed with treatment groups.

A)



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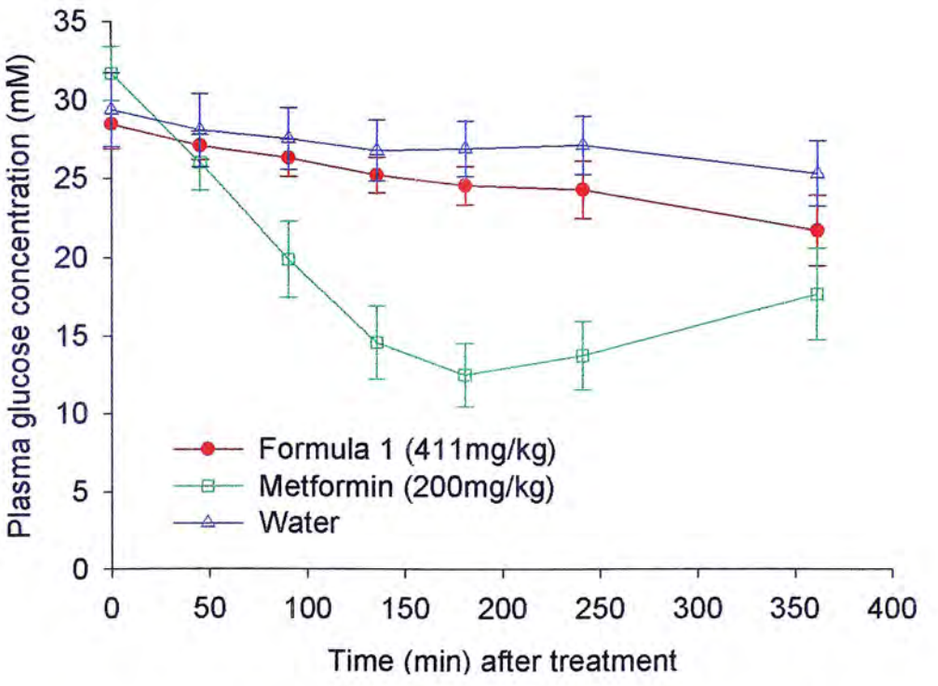


Figure 4.4. Basal glycaemia test of formula 1 (411mg/kg) treated animals on (A) day 1 and (B) day 8 experiment. Formula 1 (411mg/kg), or metformin (200mg/kg), or water, was administered orally to the n5-STZ diabetic rats after 2hr fasting (t = 0). Blood was taken at 45, 90, 135, 180, 240 and 360min after treatment. The treatment lasted for 8 consecutive days and basal glycaemia test was performed on day 1 and day 8 of the treatment. Data are expressed as mean $\pm$ SEM (n = 5 - 7).

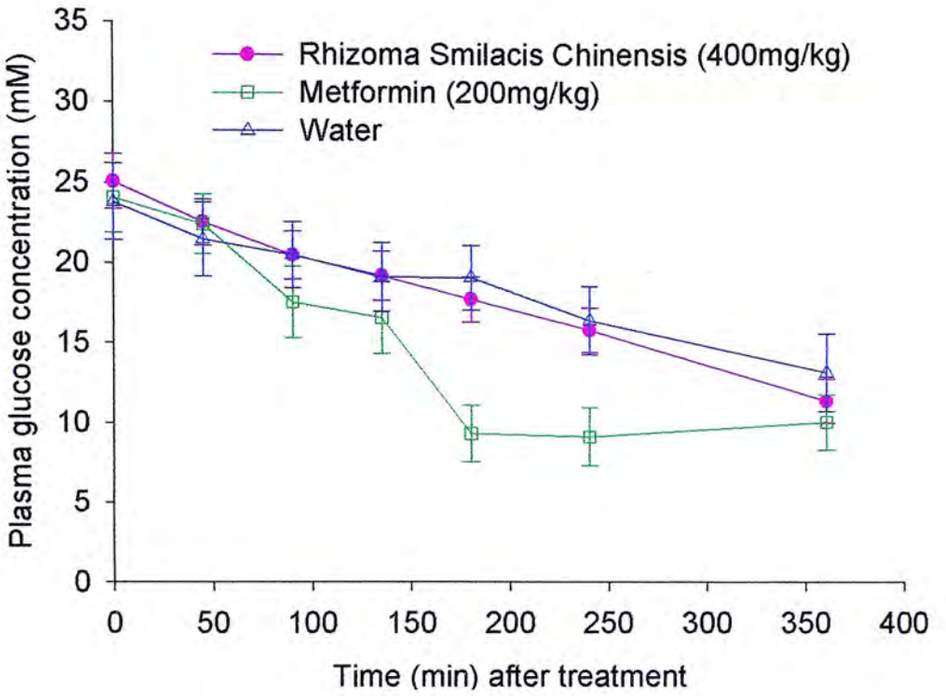
b) Rhizoma Smilacis Chinensis (菝葜)

Rhizoma Smilacis Chinensis was tested for its effect on fasting glucose level by basal glycaemia test in n5-STZ diabetic rats and the results are shown in Figure 4.5. The dosage of Rhizoma Smilacis Chinensis used in the experiment was 400mg/kg. Metformin at the dosage of 200mg/kg was used as positive control. Statistical analysis was made by comparing area under the curve (AUC) of treatments with that of the water control.

On both days 1 and 8, no significant difference from water group was found in Rhizoma Smilacis Chinensis treatment group. On the other hand, the positive control, metformin, significantly lowered the plasma glucose level on both days ($p<0.05$), thereby validating the model. Hence, it is concluded that Rhizoma Smilacis Chinensis at the dosage of 400mg/kg did not have glucose-lowering effect on fasting glucose on the n5-STZ rats.

After 8 days of treatment, body weights of rats in all groups increased gradually and no significant difference was found between treatment groups and water group (data not shown). No obvious side effect was observed with treatment groups.

A)



B)

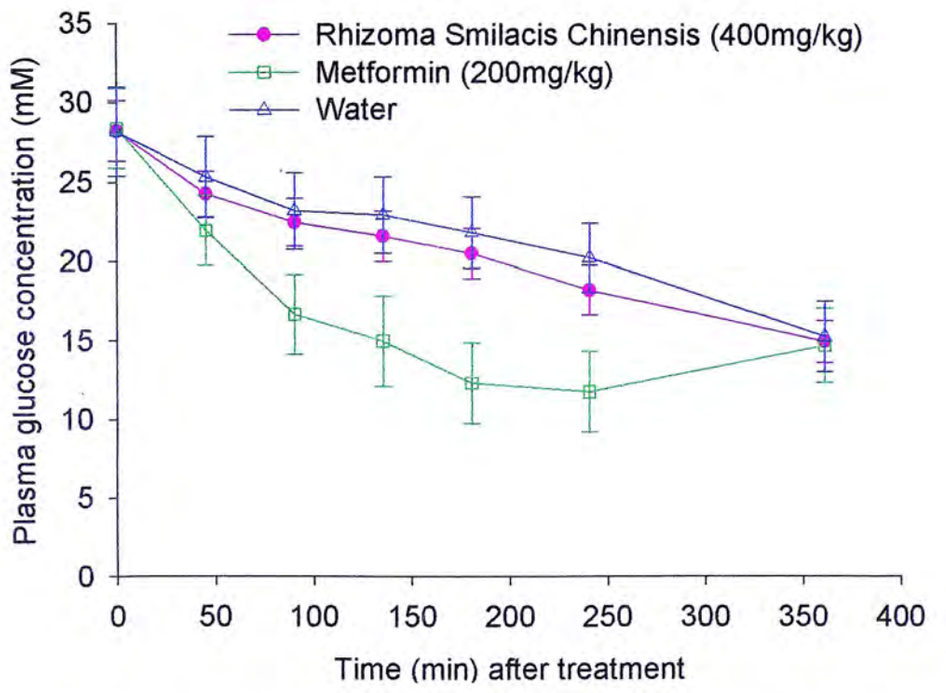


Figure 4.5. Basal glycaemia test of Rhizoma Smilacis Chinensis (400mg/kg) treated animals on (A) day 1 and (B) day 8 experiment. Rhizoma Smilacis Chinensis (400mg/kg), or metformin (200mg/kg), or water, was administered orally to the n5-STZ diabetic rats after 2hr fasting (t = 0). Blood was taken at 45, 90, 135, 180, 240 and 360min after treatment. The treatment lasted for 8 consecutive days and basal glycaemia test was performed on day 1 and day 8 of the treatment. Data are expressed as mean ± SEM (n = 12 - 13).

4.6 Discussion

The STZ diabetic rat model has long been used for the study of diabetes (Portha *et al.*, 1974). In this project, the n0-STZ rats were used in the oral glucose tolerance test while the n5-STZ rats were used in the basal glycaemia test. The severe hyperglycaemia condition created in the n5-STZ rats provides a more sensitive model for the basal glycaemia test. Basal glycaemia test was also conducted in n0-STZ rats. However, the hypoglycaemic effect observed in metformin treatment was slight. For the herbs that were expected to be not as powerful as metformin, the n0-STZ model is not sensitive enough for the detection of the hypoglycaemic effect of the herbs (Appendix II). Metformin produces hypoglycaemia in diabetic animals by an extrapancreatic mechanism and do not have significant activity in normal animals (El-Fiky *et al.*, 1996). In the present study, results showed that metformin could significantly decrease the plasma glucose level in diabetic animals in all sets of experiments (Figure 4.2 to Figure 4.5). Therefore, the diabetic condition of the animal model was confirmed.

Formula 1 and Rhizoma Smilacis Chinensis were tested for their anti-diabetic effects in the STZ diabetic rat model. However, no significant hypoglycaemic and anti-hyperglycaemic effects were observed. The dosage of formula 1 used in the animal study is equivalent to the human dose or twice of the human dose. However, the dosage for *in vivo* study of Rhizoma Smilacis Chinensis (400mg/kg) in the *in vivo* study was much higher than that of the human equivalent dose (38.8mg/kg) (State Pharmacopoeia Commission, 2000; Freireich *et al.*, 1966). Preliminary test on the human equivalent dose of Rhizoma Smilacis Chinensis did not show anti-diabetic

effect in the animal model (data not shown). Therefore, a higher dosage was used for the study of the *in vivo* anti-diabetic effect of Rhizoma Smilacis Chinensis extract.

Although formula 1 and Rhizoma Smilacis Chinensis showed promising effect in the *in vitro* screening systems, they had no significant effect in the *in vivo* systems. Such disagreement in the two systems may be due to the following: (1) The active compounds in the herbal extracts might have been broken down through digestion or these compounds were not absorbed into the bloodstream. (2) The dosage used in the *in vivo* assays did not match the effective dosage observed in the *in vitro* screening systems. It is always a difficult task to correlate the dosage used in *in vitro* systems with that in *in vivo* systems for these crude herbal extracts because these extracts contain a number of compounds, which may have different absorption efficiency. (3) The animal model has some limitations. Some consider this model representative of type 2 diabetes while some have reservations. Neonatal streptozotocin-induced diabetic rat model is the model of diabetes associated with substantial reduction in pancreatic β-cell mass and this feature is also present in diabetic patients (Stefan *et al.*, 1982). However, insulin secretion was insensitive to glucose challenge (Leahy *et al.*, 1984) and sulphonylureas (Weir *et al.*, 1981) in the STZ-induced diabetic rats, which does not occur in human. Therefore, the anti-diabetic effects of the herbs might not be detected if these herbs mimic the action of sulphonylureas. Moreover, insulin resistance is only present in the n5-STZ rats, but not in n0-STZ rats (Blondel *et al.*, 1989; Kergoat and Portha, 1985).

In the future, other diabetic animal models can be used, such as the genetically manipulated diabetic rat models, e.g. *db/db* mice. Other chemically manipulated

diabetic rat models can be used, such as the rat model induced by the injection of streptozotocin and nicotinamide, the pancreatic β-cell protective agent, which showed sensitivity to sulphonylureas (Masiello *et al.*, 1998). However, it must be noted that none of the current diabetic animal models are equivalent to the human condition (Bell and Hye, 1983).

From section 2.5.3, it was found that the herbal extracts contained glucose, fructose and sucrose. In this regard, one may be concerned that the presence of the sugars may affect the plasma glucose level. However, if the amounts of these sugars were low, the plasma glucose level would not be affected. The amount of sugars in formula 1 and Rhizoma Smilacis Chinensis extract was calculated and is shown in Table 4.1. Glucose released from the herbal extracts should come from the free glucose and sucrose. Sucrose releases glucose and fructose at 1:1 ratio under digestion. Concerning oral glucose tolerance test, 2000mg/kg of glucose was administered to the rats. The amount of glucose released from formula 1 (822mg/kg) and Rhizoma Smilacis Chinensis (400mg/kg) was responsible for 2.33% and 1.06% of the glucose load, respectively. Concerning basal glycaemia test, the amount of glucose released from formula 1 (411mg/kg) and Rhizoma Smilacis Chinensis (400mg/kg) was at a dosage of 23.33mg/kg and 21.28mg/kg, respectively. Therefore, it is believed that the glucose released from the herbal extracts would not interfere with the *in vivo* test results.

In conclusion, formula 1 and Rhizoma Smilacis Chinensis did not show anti-hyperglycaemic and hypoglycaemic effects *in vivo*. In the next chapter, the effects of formula 1 and formula 2 on erythrocyte glucose uptake and fasting plasma glucose levels in diabetic patients receiving the formulae treatment are discussed.

	Dosage	Amount of glucose received by the rats (mg/kg body weight)		
		D-Glucose	Glucose released from sucrose	Total
Formula 1	822mg/kg	16.93	29.72	46.65
	411mg/kg	8.47	14.86	23.33
Rhizoma Smilacis Chinensis	400mg/kg	21.28	0	21.28

Table 4.1. The amount of glucose received by the rats in the *in vivo* study. The amount was calculated from the HPLC analysis of the sugar content in the herbal extracts in section 2.5.3 and the dosage used in the animal study.

Chapter 5: The effects of the TCM treatment on glucose homeostasis in diabetic foot ulcer patients

5.1 Introduction

In type 2 diabetes, hyperglycaemia is the main feature and normal glucose homeostasis cannot be maintained due to insulin resistance and impaired insulin secretion. It was found that reduction in both insulin-stimulated glucose uptake and basal glucose uptake play important role in the development of diabetes (Forbes *et al.*, 1998).

Glucose transporter 1 (Glut1) is present in nearly all tissues and plays pivotal roles in basal glucose uptake (Mueckler, 1990). In diabetes, the basal glucose uptake in the patients is diminished and reduction of the intrinsic activity of Glut1 accounts for such defect (Comi and Hamilton, 1994). Glut1 is most abundant in the erythrocytes and it is the only type of glucose transporter present on the erythrocyte membrane. Erythrocytes transport 40% of blood glucose and it is the most important tissues after the brain in terms of glucose consumption (Jacquez, 1984). Glucose transport and metabolism were reported to be reduced in type 2 diabetic patients (Donatelli *et al.*, 1991), which the reduced Glut1 activity on the erythrocyte membrane is due to the Glut1 structural change (Hu *et al.*, 2000).

Owing to the lack of nucleus in erythrocytes, the high content of Glut1 in erythrocyte membrane is steady and there is no new protein biosynthesis. Therefore, the erythrocyte is a good model to study the activity of Glut1 protein. Using erythrocyte as a model of basal glucose transport, Yoa *et al.* (1993) demonstrated

that a defect in glucose uptake exists in erythrocytes from diabetic patients and this defect is reversed by metformin which is known to promote basal glucose transport in diabetes (Kumar and Dey, 2002).

In this part of the project, the effect of formula 1 and formula 2 treatment on basal glucose transport was studied in diabetic patients. Patients were recruited and subjected to TCM or placebo treatment on a double-blind basis. Blood samples were taken from the patients and zero-trans influx glucose uptake experiment in the erythrocytes was performed to determine Glut1 activity. Comparison was made between the erythrocyte glucose uptake before and after treatment. The effects of the treatment on the fasting plasma glucose concentration of the patients were also studied.

5.2 Objective

The objective of this part of the project is to study the effects of formula 1 and formula 2 treatment on systemic and cellular glucose homeostasis by measuring fasting plasma glucose level and erythrocyte glucose uptake in diabetic patients.

5.3 Materials

5.3.1 Study subjects

Diabetic foot ulcer patients were recruited to the project investigating the effect of the TCM formulae, formula 1 and formula 2, on the healing of diabetic foot ulcer. The patients were recruited from Prince Wales Hospital, Hong Kong and these patients were diagnosed as diabetes complicated with diabetic foot ulcer (Leung *et al.*, unpublished data). As most of the patients had a long duration of diabetes, they received anti-diabetic medication during the formulae study.

Patients admitted to the project were under formulae treatment on a random double-blind basis, indicating that approximately half of the patients received TCM formulae treatment and half of them received placebo. Placebo were made with cellulose and coloured to the same as the herbal extract, identical in appearance, smell, taste and weight with the formulae. Apart from double-blind basis, some patients were recruited as service cases, which means that they are not part of the double-blind basis clinical trial and they were given TCM formulae for ulcer treatment. In total, eight patients were recruited into the randomized clinical study, which were designated as "study case". At the end of the study, the treatment received by each patient was revealed and it was found that four cases were in TCM formulae treatment group and four cases in the placebo group. Moreover, three patients were recruited as "service cases" and they received TCM formulae treatment group. Therefore, seven patients received TCM treatment and four patients received placebo treatment. Blood was taken from the patients before the start of the therapy and after the end of the therapy, which the patients stopped receiving treatment.

5.3.2 Blood sample

Blood is preserved at 4°C in coagulation tubes containing 3.2% (w/v) sodium citrate. The glucose uptake assays were performed within 2 days after the blood was taken.

5.3.3 Chemicals and reagents for erythrocyte glucose uptake assay

(a) 3-O-Methyl-D-gluco-pyranose (3-OMG)

3-OMG was purchased from Sigma (St. Louis, MO, USA). The powder was dissolved in PBS as a stock solution of 10mM and stored at 4°C until use.

(b) ^{14}C-labeled 3-OMG

The stock solution (56.4mCi/mmol) was purchased from PerkinElmer Life Science, Inc. (Boston, MA, USA) and kept at -20°C.

(c) Stop solution

The stop solution contained 100μM mercuric chloride and 50μM phloretin in ice-cold PBS, prepared immediately before use.

(i) Phloretin

Phloretin (aglycone of phlorizin) was purchased from Sigma (St. Louis, MO, USA). It was freshly prepared by dissolving 13.75mg of phloretin powder in 0.5ml 95% ethanol.

(ii) Mercuric chloride

Mercuric chloride ($HgCl_2$) was purchased from Sigma (St. Louis, MO, USA). It was freshly prepared by dissolving 27.15mg mercuric chloride powder in 1ml of PBS.

(d) Cell solublizing agent

Cell solublizing agent was prepared by mixing isopropyl alcohol (Sigma, St. Louis, MO, USA) and soluene-350 (Packard Bioscience, Meriden, CT, USA) in the ratio of 1:2.

(e) Hydrogen peroxide 30% (v/v) (H_2O_2)

H_2O_2 was purchased from BDH Laboratory (Dorset, United Kingdom) and stored at 4°C.

5.4 Methods

5.4.1 Preparation of blood sample

One to two millilitre of blood was washed twice by 5 volume of PBS and centrifuged at 805 x g for 4 minutes at 4°C. Supernatant was discarded, and erythrocytes were then incubated with PBS on wet ice for 15 minutes to achieve zero-trans (no intracellular glucose) condition, followed by centrifugation and the removal of supernatant. Erythrocytes were then re-suspended in PBS to the original blood-sample volume. Fifty microlitres aliquots were taken for immediate assay, and 10μl were used for cell count.

5.4.2 Zero-trans influx of 3-OMG uptake in erythrocytes

The Lowe and Walmsley techniques were modified as previously described (Klepper *et al.*, 1999; Lowe and Walmsley, 1986). Briefly, 50μl of blood aliquot was incubated with 100μl of hot/cold 3-OMG mixture (H/C solution) containing 1μCi/ml of ^{14}C-3-OMG and 0.6mM of cold 3-OMG. The uptake reactions with ^{14}C-labeled 3-OMG were allowed to proceed at 4°C and the reaction was stopped at different time points over a period of 30 seconds. Equilibrium uptake of ^{14}C-labeled 3-OMG was measured after 25 minutes incubation. 3-OMG influx was terminated by addition of 1ml ice-cold stop solution followed by the washing steps carried out at room temperature. After centrifugation for 20 seconds, the erythrocyte pellet was washed twice with 1ml of ice-cold stop solution. Cell pellet in each tube was then digested with 250μl of solublizing agent. At room temperature, the samples were bleached with 250μl of 30% v/v hydrogen peroxide. The mixture was swirled until all foaming

subsided. Four milliliters of OptiPhase HiSafe 2 scintillation fluid (PerkinElmer Life sciences, Inc., Boston, MA, USA) was added to each sample, and radioactivity in aliquots was determined by a Packard Tri-Carb® 2900TR liquid scintillation counter.

5.4.3 Statistical analysis

For the statistical analysis, data were entered into and analyzed by using SPSS for Windows (version 11.5, SPSS Inc, Chicago, IL, USA). Mann-Whitney tests were used for the comparisons in all experiments between the data before and after treatment in both TCM treatment and placebo groups. Statistical tests were two-sided, with a significant level of 0.05.

5.5 Results

In the present study, eleven patients were tested for the effects of the formulae or placebo on fasting plasma glucose concentration and erythrocytes glucose uptake. The profiles of the patients are shown in Table 5.1. Seven of them received TCM formulae treatment and four of them received placebo treatment.

The fasting plasma glucose concentration and the erythrocytes glucose uptake were measured before and after the course of treatment. Figure 5.1 shows the fasting plasma glucose concentration of the patients in both treatment and placebo groups. When considering the fasting plasma glucose concentration of individual patients, although drastic changes were observed in some patients, the average glucose concentration in both groups did not change significantly (Figure 5.1A, B and C). Mild decrease of fasting plasma glucose level was observed in TCM treatment group, but such decrease did not reach statistical significance (Figure 5.1D).

The erythrocyte glucose uptake of the patients is shown in Figure 5.2. Similarly, both TCM formulae treatment and placebo treatment did not significantly change the erythrocyte glucose uptake activity. The differences observed in all the treatment group did not reach statistical significance.

		Sample size (N)	Age	Gender	Treatment duration (Weeks)
TCM group	**Study case**	4	78.5 ± 4.1	M = 2 F = 2	8.0 ± 3.3
	Service case	3	60.7 ± 10.9	M = 1 F = 2	8.3 ± 4.5
Placebo		4	69.7 ± 1.2	M = 0 F = 4	3.5 ± 0.6

Table 5.1. The sample size, age and gender distribution of the patients in TCM formulae treatment group and placebo treatment group.

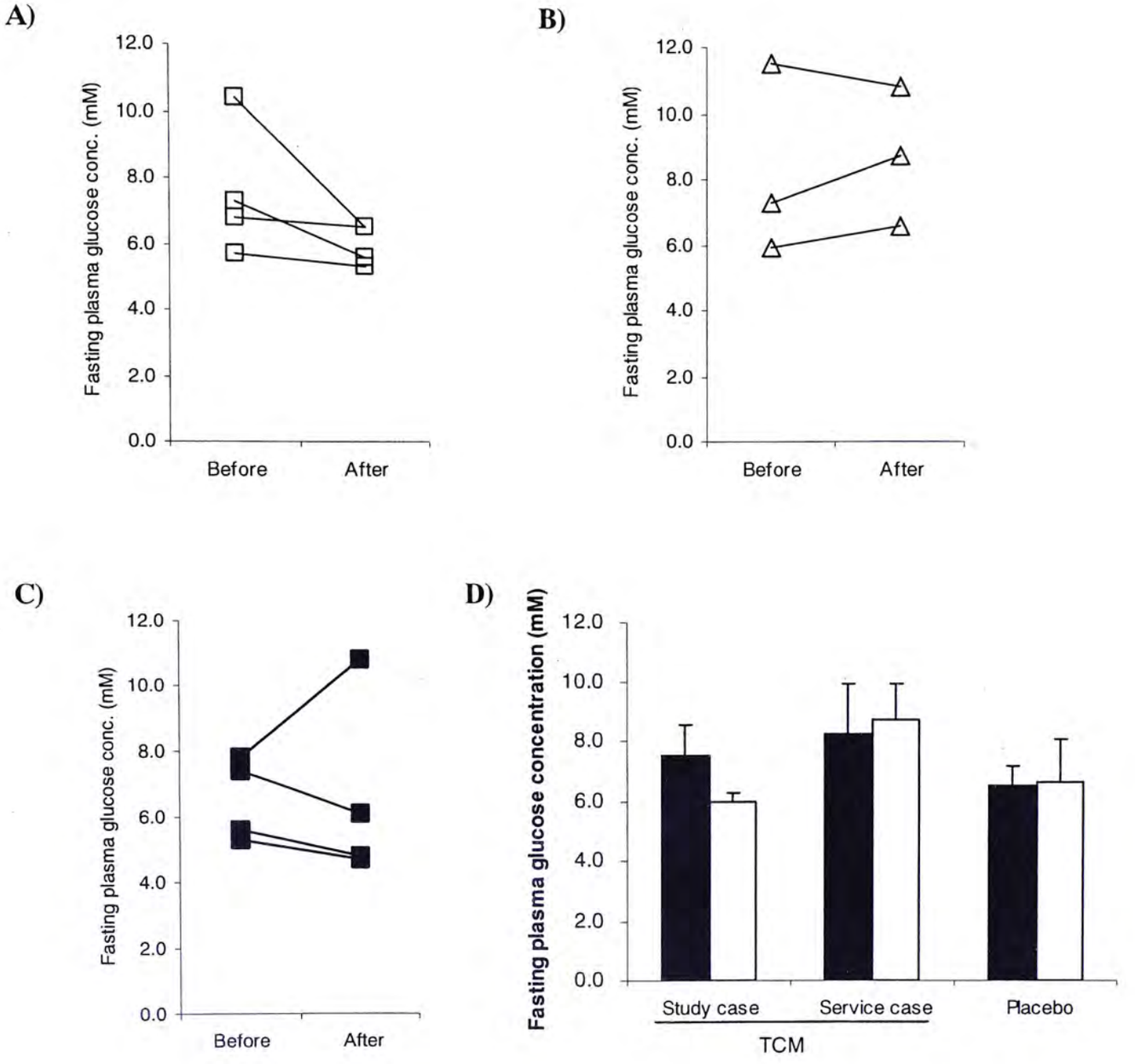


Figure 5.1. Fasting plasma glucose concentrations of patients before and after (A) TCM formulae treatment in study case, (B) TCM formulae treatment in service case and (C) placebo treatment, and (D) the average values in each treatment group. The fasting plasma glucose concentration was measured after overnight fasting. Data are expressed as mean + SEM (n = 4 for TCM treatment group in study case; n = 3 for TCM treatment group in service case; n = 4 for placebo group).

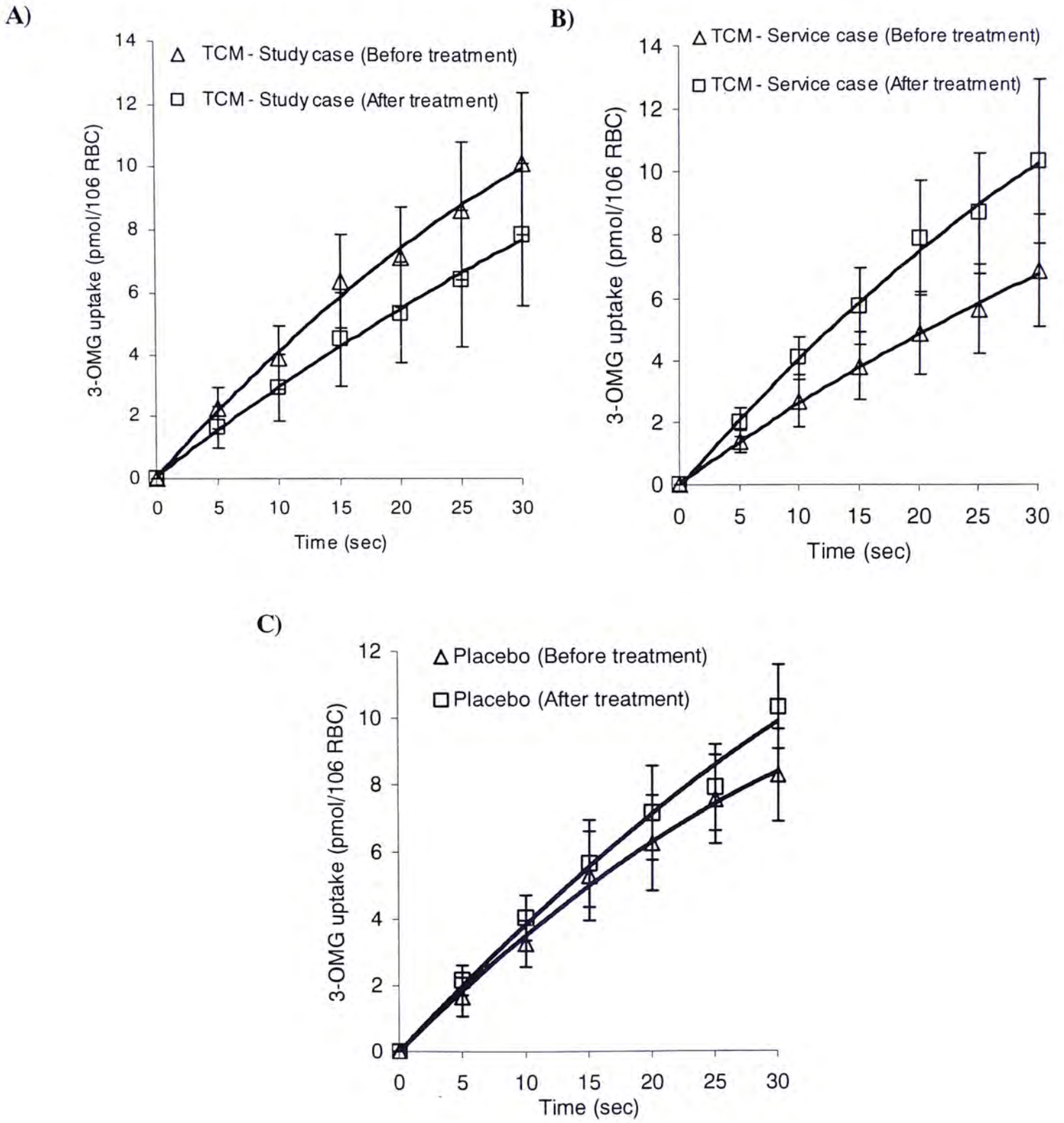


Figure 5.2. The erythrocyte 3-OMG uptake of the patients before and after (A) TCM formulae treatment in study case, (B) TCM formulae treatment in service case and (C) placebo treatment. Erythrocytes were incubated with ^{14}C-3-OMG at 4°C. Reaction was stopped at different time points over a period of 30 sec. 3-OMG influx was terminated by the addition of ice-cold stop solution. The amount of radioactive glucose influx was normalized by cell count. Data are expressed as mean ± SEM (n = 4 for placebo group; n = 7 for TCM treatment group).

5.6 Discussion

In this study, the formulae treatment did not improve the fasting plasma glucose value and the erythrocyte glucose uptake in diabetic patients. With regards to the rationale of this project, the anti-diabetic effects of the formulae were studied because the patients were prescribed with these formulae, as well as conventional anti-diabetic medications, and it may cause hypoglycaemia in patients if these formulae also possess strong hypoglycaemic effect. In the present study, it is demonstrated that these formulae do not have significant hypoglycaemic effect on the patients.

The erythrocyte glucose uptake was not improved by the formulae treatment in patients. This may also be due to the parallel formulae treatment and anti-diabetic medications, masking the effect of the formulae treatment. From previous report, metformin treatment can restore the defective erythrocyte glucose uptake in diabetic patients (Yoa *et al.,* 1993; Rapin *et al.*, 1991). When compared with the erythrocyte glucose uptake in normal subjects (Wong *et al.*, unpublished data), the uptake in diabetic patients in this study was not significantly changed when compared to that of normal. However, other studies have shown that the erythrocyte glucose uptake in the diabetic patients is only about 60% of the normal (Hu *et al.*, 2000; Comi and Hamilton, 1994). This indicates that the anti-diabetic medication may have improved the erythrocyte glucose uptake in these patients through glycaemia control. Moreover, since the turnover rate of erythrocytes is about 110 days (Strocchi *et al.*, 1992) and the patients seldom received the formulae treatment for such long time, the lack of effect of the formulae treatment on erythrocyte glucose uptake may be

due to the insufficient time for the actions of the formulae to be reflected on erythrocyte Glut1 activity.

The studies are still ongoing at this moment. Moreover, owing to the very small sample size, the results were preliminary and further study is needed to confirm the effects of the formulae treatment on systemic glucose homeostasis in human. More information about the glycaemic control of the patients will be measured in the future, e.g. HbA1c, plasma insulin and TNF-α level. At present, the formulae treatment did not show significant effects on regulating plasma glucose level or erythrocyte glucose transport in diabetic patients.

Chapter 6: General discussion and conclusion

6.1 Overview of the project and analysis of research findings

Type 2 diabetes mellitus is a major health burden in the world. The prevalence of diabetes surges dramatically in recent years (Lieberman, 2003). However, current therapies have limited efficacy, limited tolerability and significant side effects (Moller, 2001). Therefore, the search for new anti-diabetic agents is much needed. Medicinal plants have always been one of the main research interests and new anti-diabetic agents may be discovered from these traditionally used herbs.

托毒生肌顆粒劑 (Formula 1) and 耆味地黃顆粒劑 (Formula 2) are two traditional Chinese medicine formulae designed for diabetic foot ulcer treatment. In this project, anti-diabetic activities of these traditional Chinese medicine formulae were systematically studied. Because it is a possible mechanism contributing to the therapeutic effects of formulae on diabetic foot ulcer. It was hypothesized that these formulae and their component herbs possess anti-diabetic activities. The main focus of this project is on formula 1 while formula 2 and its component herbs have been tested by the other member of this project.

Before the herbs were used in the experiments, morphological studies were employed to authenticate these herbs and their authenticities were further supported by their thin layer chromatographic profiles. The anti-diabetic activities of formula 1 and its component herbs were studied using both *in vitro* and *in vivo* models (Figure 6.1). The *in vitro* systems were employed to firstly screen the effects of the herbal extracts on modulating tissue glucose homeostasis. The herbs showing potent effects

in the *in vitro* screening systems were further studied for their *in vivo* anti-diabetic effects by using the neonatal-STZ diabetic rat model. Furthermore, the effects of the formulae treatment on the fasting plasma glucose level and the erythrocyte glucose transport were also investigated in diabetic patients.

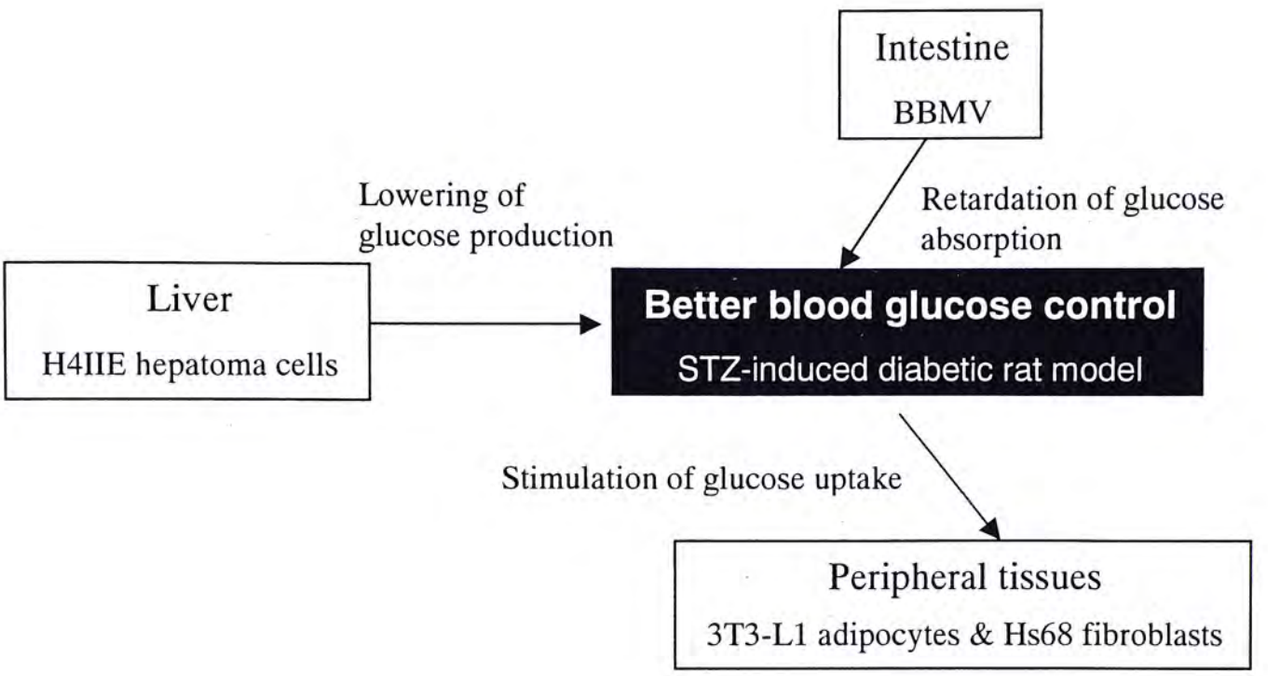


Figure 6.1. The systematic approach used in this project for the investigation of the anti-diabetic activities of formula 1 and its component herbs. Four *in vitro* models were employed in the screening process: 3T3-L1 mouse adipocytes and Hs68 human skin fibroblasts were used as the models to study the effect of herbal extracts on peripheral tissues glucose transport. H4IIE rat hepatoma cells were used as the model to study the effects of the herbal extracts on hepatic gluconeogenesis. BBMV was used as the model to study the effects of the herbal extracts on intestinal glucose absorption. The anti-diabetic effect of the herbs on systemic blood glucose was studied by using the STZ-induced diabetic rat model.

Significant findings of this project include:

(1) The extracts of formula 1 and its component herbs were found active in modulating tissue glucose homeostasis in the *in vitro* studies.

(2) Formula 1 and Rhizoma Smilacis Chinensis did not exert anti-hyperglycaemic or hypoglycaemic effects in the *in vivo* animal studies.

(3) Formula 1 and formula 2 treatment did not significantly improve fasting plasma glucose level or erythrocyte glucose uptake in the diabetic patients in the clinical studies.

The formulae showed prominent effects on treating diabetic foot ulcer, but neither formula 1 nor its component herbs significantly lowered blood glucose level in patients or in animal studies. Nevertheless, these herbal extracts did modulate tissue glucose homeostasis *in vitro*. These observations indicate that the therapeutic effects of formula 1 on foot ulcer are not primarily mediated by normalizing systemic glycaemic control *in vivo*. In TCM theory, the treatment of diabetes emphasizes the relief of the symptoms and restoration of the inner-balance of body rather than the lowering of blood glucose (Zhou, 2001). Therefore, formula 1 may not exert its effect by directly lowering the blood glucose level. Rather, the therapeutic effect on diabetic foot ulcer of formula 1 may be mediated by the improvement of the peripheral tissue glucose homeostasis as shown in the *in vitro* studies.

Diabetic foot ulcer is a complication of diabetes mellitus. Common treatment mechanisms include anti-inflammation, tissue generation and angiogenesis (Mason *et al.*, 1999). Formula 1 was designed to treat diabetic foot ulcer through strengthening

of the muscle, swelling control, removal of the debridement and promotion of wound granulation. The effects of the formulae on diabetic foot ulcer are studied by another project team member. According to previous studies, some of the component herbs are reported to possess tissue generative effect (Radix Astragali), anti-inflammatory effect (Radix Rehmanniae) and enhancing effect on circulation (Rhizoma Smilacis Chinensis and Radix Polygoni Multiflori Preparata) (Lu *et al.*, 1999; State Administration of Traditional Chinese Medicine, 1999). These herbal effects may be the mechanisms underlying the therapeutic effects of formula 1 on foot ulcer. Since formula 1 was not designed for treating diabetes, it is not surprising that this formula does not improve *in vivo* glycaemic control.

Inconsistent results were observed in the *in vitro* and *in vivo* investigation of the herbs. Formula 1 and Rhizoma Smilacis Chinensis regulated cellular glucose homeostasis in the *in vitro* systems but they did not lower glucose level in the *in vivo* studies. This inconsistency may be due to the amount of the active compounds absorbed into the systemic circulation in the *in vivo* study did not reach the effective dosage observed in the *in vitro* screening systems. Moreover, the active compounds in the herbal extract might have been degraded in the digestive tract. The formulae did not exert significant effects on glucose homeostasis in the clinical studies either, possibly due to the parallel anti-diabetic medication received by the diabetic patients.

From the work of my fellow, Mr. Lau Chun Hong (M.Phil. candidate, Institue of Chinese Medicine, The Chinese University of Hong Kong), who studied the anti-diabetic activities of formula 2, results also showed that formula 2 can modulate *in vitro* glucose homeostasis but it did not improve the glycaemic control in the *in vivo*

studies. Although both formula 1 and formula 2 did not show hypoglycaemic effects in the *in vivo* studies, the results may indicate that these formulae give benefits to the clinical studies. In the clinical studies, the patients received both formulae and conventional anti-diabetic medication treatment. If the formulae possessed glucose lowering effects, it would cause undesirable hypoglycaemia in the patients. From our results, we can tell that it is safe for the patients to receive both formulae and medication treatment without worrying about the side effect of hypoglycaemia.

6.2 Limitations of the study

Herbal medicines were used as the only way of treatment for diabetes in ancient China. Although scientific background of the use of these herbs is insufficient, it is believed that some of these herbal medicines possess anti-diabetic activity. In the present study, one TCM formula and its six component herbs were studied for their anti-diabetic activities. However, quality control and standardization of these herbal medicines are always a concern for TCM research. The chemical composition and the efficacy of these herbal medicines are affected by a lot of factors, from agricultural conditions to storage. To limit this variability, a single batch of herbal material was used for extract preparation. However, the positive effect shown in the present study is not necessarily reproducible in another batch of the same species of herbs. This is the intrinsic limitation of herbal medicine research and can be solved by the standardization of TCM through measures like good agricultural practice (GAP) (Yang *et al.*, 2003).

Moreover, in traditional Chinese medicine theory, formulae containing more than one herb are often used for treatment. The effect of each component herbs may

be very mild in the formula (Meng *et al.*, 2000). Interactions between component herbs, like synergistic effect and neutralization of toxicity, may be present (Zhang and Wumanjiang, 2003). However, the study of the action of each component herbs in the formula is the most simple and effective way to isolate the active components in the formula.

Traditional Chinese herbs are usually boiled with water as decoction for medicinal use. Compliance to this traditional use of Chinese herbs, water extracts of the herbs were used for investigation in this project. However, water extraction inevitably leads to the loss of non-polar compounds in the herbs, e.g. alkaloids and flavonoids. Although the anti-diabetic activity of these non-polar compounds in the herbs of this project is not known, active non-polar compounds with anti-diabetic activity have been isolated from other herbs (Miura *et al.*, 2001; Shi and Zhang, 2000). Therefore, active non-polar compounds may also be present in the component herbs of formula 1. However, their anti-diabetic activities were not studied in the present investigation.

6.3 Future directions

There are two main future directions to investigate the traditional Chinese medicines for their potential therapeutic effects on diabetes: investigation of the anti-diabetic activity of other traditional Chinese medicines and isolation of active compounds from the herbs in the present study.

Although systemic glucose-lowering effect was not observed in formula 1 and its component herbs, it is still possible that other traditional Chinese medicinal herbs

possess anti-diabetic activity because TCM has been used for diabetic treatment since ancient time. Therefore, other herbs with potential anti-diabetic activities can be investigated in the future. Ethnopharmacological approach (the study of differences in response to drugs based on varied ethnicity) should be used instead of random selection of the herbs. Ethnopharmacological approach allows recognition of potential herbs by their traditional uses in diabetes treatment and provides a more effective way with greater success rate to identify the anti-diabetic herbs (Heinrich and Gibbons, 2001). For example, Radix Trichosanthis (天花粉), Rhizoma Coptidis (黃連), Raidix Ophiopogonis (麥冬) and Rhizoma Anemarrhenae (知母) are the common ingredients of formulae used in traditional diabetes treatment (Wu and Ren, 2002) and their anti-diabetic activity can be confirmed using the screening system presented in the present study.

Another approach for further investigation of the traditional Chinese medicines for their potential therapeutic effect on diabetes is the isolation of active compounds from the herbs in the present study. Although significant glucose-lowering effects were not found in formula 1 and Rhizoma Smilacis Chinensis in the *in vivo* study, they showed promising effects in the *in vitro* screening systems. The active compound(s) responsible for their effects *in vitro* is unknown. Nevertheless, these active compounds could be effective in improving glycaemic control *in vivo*, although further investigation is required. Bioassay-guided fractionation can be used to isolate the active compounds in the herbs followed by a simple bioassay, e.g. BBMV, to identify the active fraction of the herbal extracts.

Apart from the above two main future directions, other *in vitro* studies can also be performed in the future. As mentioned in section 1.5.2, pancreas is one of the major therapeutic targets for anti-diabetic medications. Therefore, stimulation of insulin release by the herbal extracts can also be studied in the future and such *in vitro* investigation can be carried out by using insulin-secreting cell line, such as rat insulinoma RIN m5F cells (Murakami *et al.*, 1992).

6.4 Conclusion

In the present study, the traditional Chinese medicine formula used for treating diabetic foot ulcer, formula 1 (托毒生肌顆粒劑), was studied with the hypothesis that this formula and its component herbs possess anti-diabetic activities.

A systematic approach has been established to investigate the anti-diabetic activities of the traditional Chinese medicinal herbs. Our research findings indicated that formula 1 and its component herbs modulate glucose homeostasis at the tissue levels without improving systemic glycaemic control.

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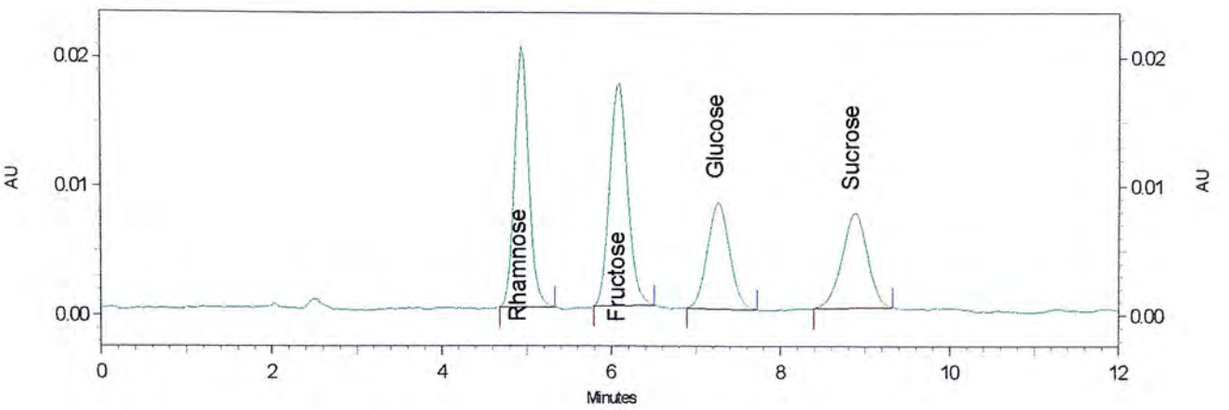
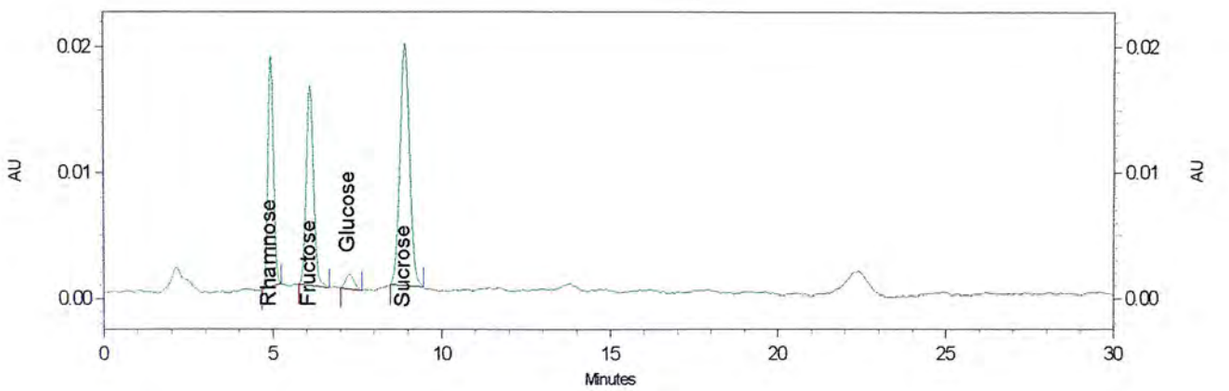
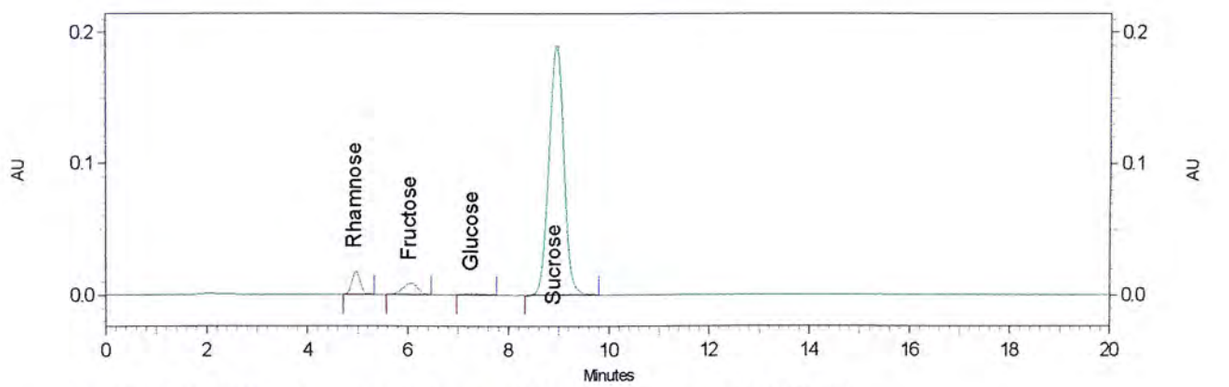
Appendix I. The determination of the sugar contents in the herbal water extracts by high performance liquid chromatography



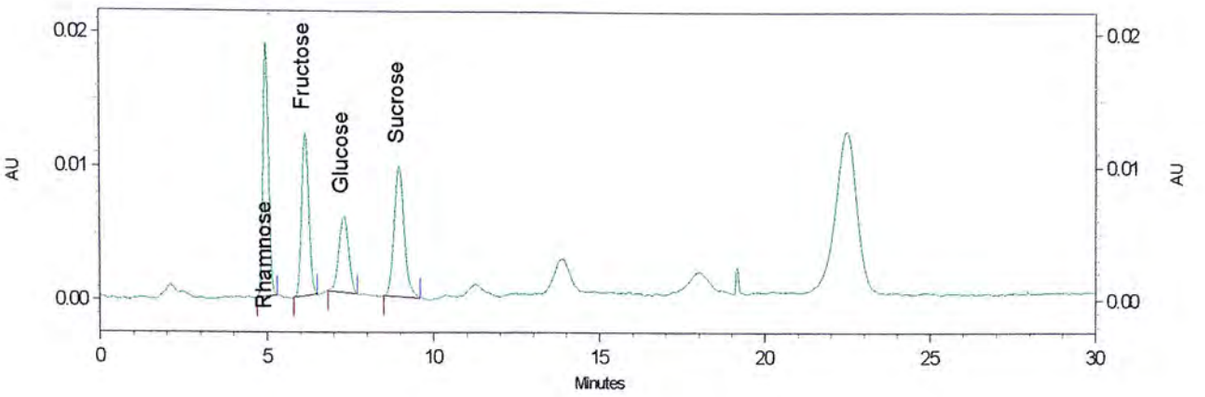
A) The HPLC chromatogram of sugar standards.



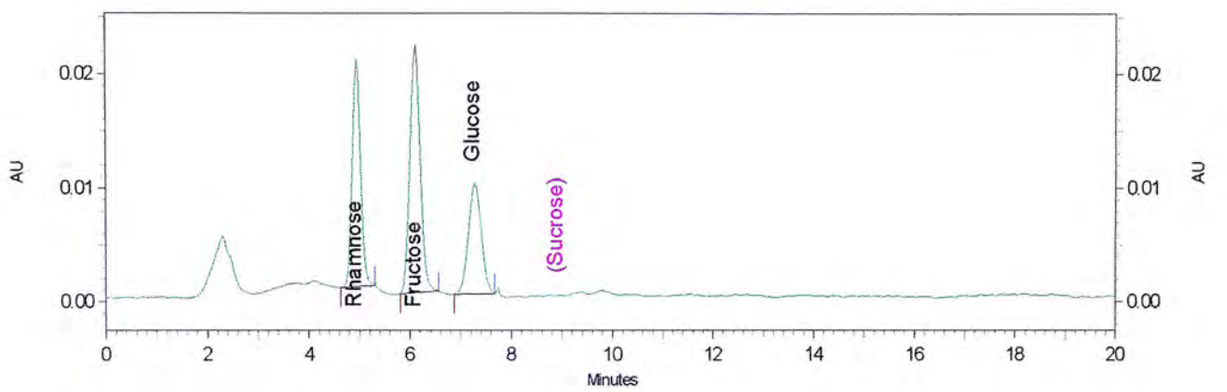
B) The HPLC chromatogram of formula 1.



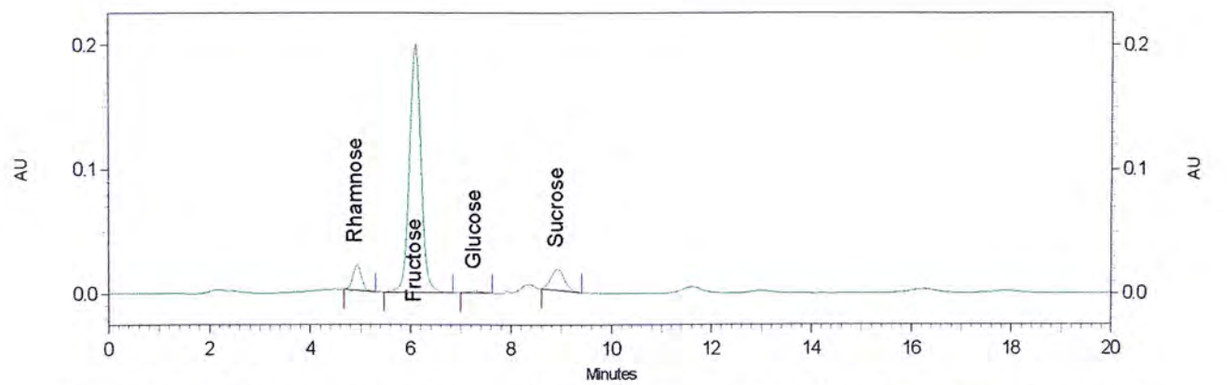
C) The HPLC chromatogram of Radix Astragali (黃耆).



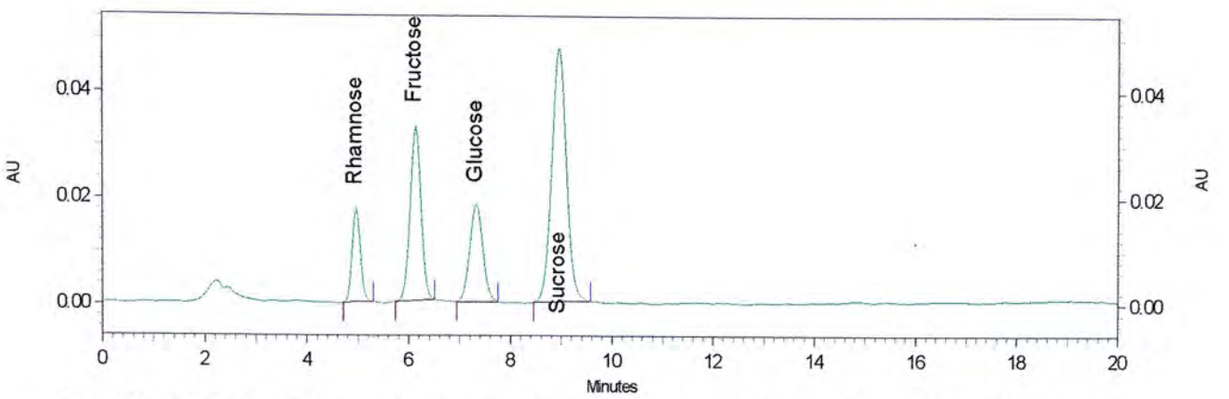
D) The HPLC chromatogram of Radix Rehmanniae (生地).



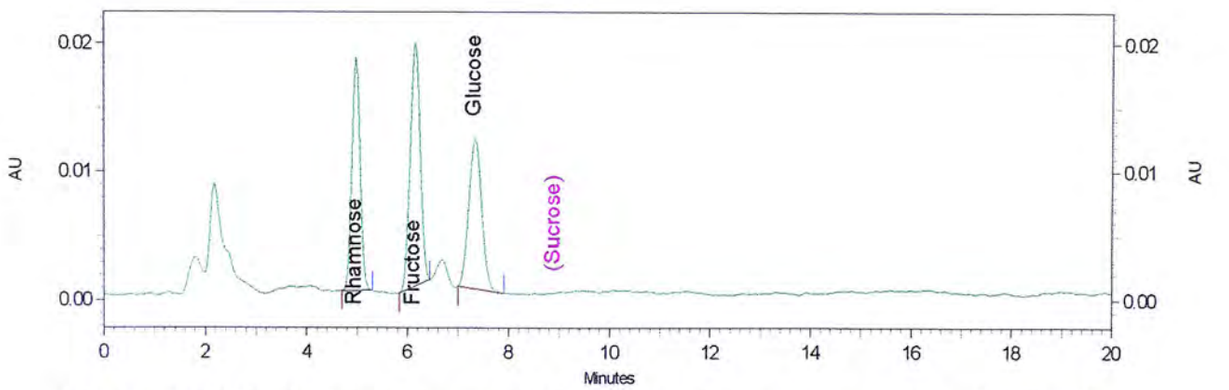
E) The HPLC chromatogram of Rhizoma Smilax China (菝契).



F) The HPLC chromatogram of Rhizoma Atractylodis Macrocephalae (白朮).



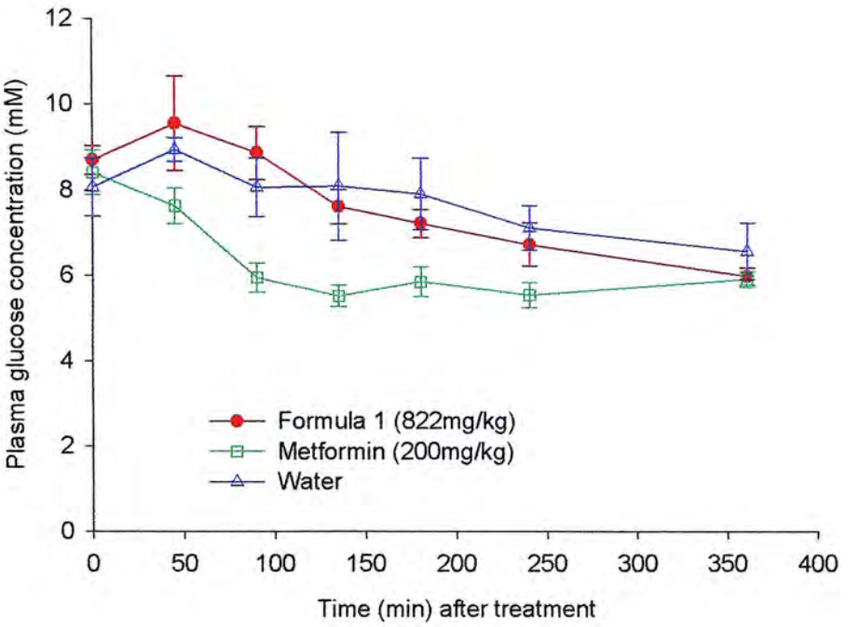
G) The HPLC chromatogram of Radix Polygoni Multiflori (制首烏).



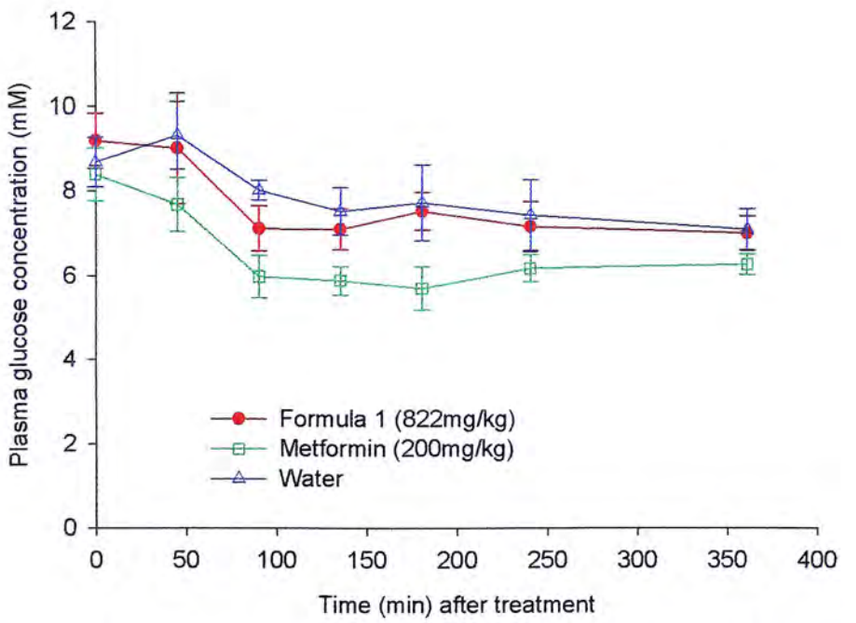
H) The HPLC chromatogram of Radix Stephaniae Tetrandrae (漢防己).

Appendix II. Basal glycaemia test of formula 1 (822mg/kg) on n0-STZ rats

A)



B)



Basal glycaemia test of formula 1 (822mg/kg) treated animals on (A) day 1 and (B) day 8 experiment. Formula 1 (822mg/kg), or metformin (200mg/kg), or water, was administered orally to the n0-STZ diabetic rats after 2hr fasting (t = 0). The treatment lasted for 8 consecutive days and basal glycaemia test was performed on day 1 and day 8 of the treatment. Data are expressed as mean ± SEM (n = 6 - 7).